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Studies on aspects of the chemotherapeutic control of the salmon louse *Lepeophtheirus salmonis* Krøyer 1837 (Copepoda: Caligidae).

by

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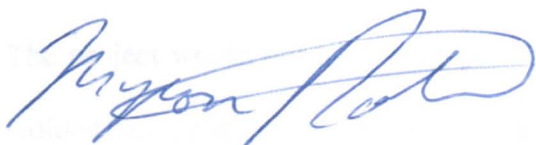
October 1992

To my dearest wife Vicki, for your encouragement and help and taking care of everything else when I was away or too caught up in my work.

Declaration

I declare that this thesis embodies results of research conducted by myself, during the period September 1989 - October 1992 while attending the Institute of Aquaculture, University of Stirling. All previous work consulted has been cited, and where appropriate, collaborative and/or technical help has been acknowledged. This work has not been submitted for any other degree.

Myron Roth

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ABSTRACT

The efficacy of a range of anti-parasitic chemotherapeutic agents against the salmon louse *Lepeophtheirus salmonis* following topical application was studied *in vitro* and *in vivo*. In general, adult and preadult lice were susceptible to a wide range of compounds with dose rates, following 1 hour exposures, ranging several orders of magnitude (10.0 - 0.001 mg/L). Overall the pyrethroid compounds which were tested were found to have the widest therapeutic ratios, indicating the potential of this group of chemotherapeutants for sea lice control. Resistance to the organophosphorus (OP) compounds dichlorvos and azamethiphos was detected in isolated populations of lice. Field trials with azamethiphos indicated that the compound was highly efficacious against sensitive lice (@ 0.1 mg/L; however, where resistance was present, efficacy (@ 0.2 mg/L) was highly variable. When used at the above dose rates, azamethiphos was found to be well tolerated by fish as indicated by a lack of significant brain acetylcholinesterase inhibition. Results on cross resistance (between pyrethroids and OPs) were inconclusive which was believed to be, primarily, due to the overall high toxicity of the group; but also to the variable responses from exposed lice. In a series of preliminary trials, one of the pyrethroid compounds, PHRDL-D, was found to effectively remove lice when administered orally to infected salmon, indicating the potential of pyrethroids as oral chemotherapeutants. A comparison of the relative toxicity of azamethiphos (OP), resmethrin (pyrethroid), ivermectin (avermectin) and the structurally similar compound SKB7 (milbemycin), indicated that chalimus stages were only susceptible to ivermectin and SKB7 following topical and intra-peritoneal injection to lice infected fish. In contrast, azamethiphos and resmethrin were found to be non toxic to chalimus larvae at dose rates which were highly toxic to both adult lice and

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treated fish. Preliminary studies on the uptake of [^{14}C]azamethiphos in adult lice indicated that uptake was both concentration and time dependant, reaching a plateau at the onset of toxicity. Uptake appeared to be primarily associated with frontal plates, 1st antennae and anus. The findings indicated that several compounds/compound classes are highly active against lice and, given the limited number of compounds available for sea lice control and the development of resistance to OPs, might be considered as alternatives. In light of these findings, the potential of chemotherapy for the future control of sea lice is discussed.

1   General Introduction

In terms of economic importance 'sea lice' are undoubtedly the most problematic

parasite in salmon mariculture. The term 'sea lice' is used here loosely to describe

several species (see Table 1.1) from the genera *Lepeophtheirus* and *Caligus* (Copepoda:

Caligidae) that parasitize cultured fishes, resulting in severe clinical pathology.

Lepeophtheirus salmonis, usually referred to as the salmon louse, is mainly restricted to

salmonid hosts and has been found to be most problematic in European salmon farms.

However, several species from the more widespread *Caligus* regularly infect salmonids

and, not uncommonly, other cultured marine fish species. Where fish are farmed at

sea, the incidental presence of sea lice is common. In many instances, such parasitism

does not result in serious pathology. However, once established, parasite numbers

slowly increase over time, eventually reaching epidemic proportions leading to the

serious sea lice problems experienced where intensive salmon farming has been

established for many years. Table 1.1 lists the geographic distribution of sea lice

reported in marine aquaculture and illustrates the widespread potential of these

damaging parasites.

Sea lice are commonly found in wild fish populations (Templeman 1967; Romero &

Kuroki 1982; Radhakrishnan & Nair 1983; Nagasawa 1987; Neilson, Percy, Scott &

Valerio 1987). Wild fishes infected by sea lice harbour relatively small parasite

burdens with little damage resulting from the parasite association. However, epidemic

outbreaks, although rare, have resulted in high mortalities in wild stocks (White 1940;

Panasenko, Jukhimenko & Kaplanova 1986), with increasing concern over the possible

effects salmon farming may have to increase wild lice populations and the resulting

Table 1.1. Geographic and taxonomic distribution of parasitic copepods and branchiurians recorded in marine fish farming (with reference to the genera *Argulus, Caligus, Ergasilus, Lepeophtheirus* & *Pseudocaligus*).

Country	Species Present	Host	Reference
Europe			
Norway	*C. elongatus, L. salmonis*	salmonids	Johannessen 1974; Håstein & Bergsjø 1976; Brandal & Egidius 1977; 1979; Høy & Horsberg 1991
Sweden	*Caligus sp.*	salmonids	Lundborg & Ljungberg 1977
Scotland	*C. elongatus, L. salmonis*	salmonids	Rae 1979; Wootten, Smith & Needham 1982
Ireland	*C. elongatus, L. salmonis*	salmonids	Tully 1989
France	*C. minimus*	sea bass	Paperna 1980
Israel	*C. pageti, E. lizae, P. apodus*	mullet	Paperna 1975
North America			
Eastern N. America	*A. alosae, Argulus sp. C. curtus, C. elongatus, E. labracis, L. salmonis*	salmonids, red drum	Hogans 1989; Hogans & Trudeau 1989a; 1989b; Landsberg, Vemeer, Richards & Perry 1991; Richard 1991; O'Halloran, Carpenter, Ogden, Hogans & Jansen 1992
Western N. America	*C. clemensi, L. cuneifer, L. salmonis*	salmonids	Johnson & Albright 1991a; 1991b; Richard 1991
South America			
Chile	*C. teres*	salmonids	Reyes & Bravo 1983
Asia			
New Zealand	*C. longicaudatus*	salmonids	Jones 1988
Malaysia	*Caligus. sp. E. borneoensis*	grouper	Leong & Wong 1988
Thailand	*C. epidemicus*	prawns	Ruangpan & Kabata 1984
Philippines	*C. patulus*	milkfish	Jones 1980
Japan	*C. orientalis, C. spinosus*	yellowtail, salmonids	Fujita, Yoda & Ugajin 1968; Izawa 1969; Urawa & Kato 1991

effect this may have on wild salmon and sea trout stocks (Tully, Poole & Whelan in press; Jakobsen, Nylund & Alexandersen in press).

The caligid life-cycle comprises of 10 life stages (see below) and is direct (Kabata, 1972). Fish are infected by a semi planktonic 'copepodid' present in the water column or from adults that may move from one host to another (Bruno & Stone 1990). Due to the continual presence of infectious copepodids, and infected wild fish that come into close contact with farm sites, farmed stock are continually at risk of infection.

The control of sea lice is one of the most intractable problems facing salmon farmers. The nature of the aquatic environment in which salmon cages are kept, possess large restriction on the control of the environment in which the salmon are grown. The largest problems being the maintenance of adequate hygiene and risks from pathogens such as sea lice present in the surrounding water. Consequently, there are few practical management techniques for sea lice control which relies on chemotherapy. In addition, due to the nature of the "fluid" environment surrounding cages, there is little control over the environmental fate of chemotherapeutic agents following use for sea lice control.

At present only two compounds have been licensed for sea lice control (Schlotfeldt 1992), dichlorvos & trichlorfon; both of which have been used successfully to control sea lice. However, there are several limitations with both dichlorvos and trichlorfon and the methods used to administer them. In addition, the potential of widespread resistance, which has already appeared in isolated populations of lice, is great further necessitating the development of alternative chemotherapeutic agents. Given the

economic importance of salmon farming worldwide and the impact that sea lice can

have, a substantial effort has been made to evaluate and develop alternative

chemotherapeutic agents for the control of sea lice. This research forms the basis of

the following study.

Although many species of caligids can be found in U.K. coastal waters (Kabata 1979) only *Lepeophtheirus salmonis* and *Caligus elongatus* are found as pests of farmed salmonids (Wootten *et al.* 1982). Thus, the following discussion will deal specifically with these two species. Where necessary, additional information will be discussed relating to other caligids and, to a lesser extent, parasitic copepods in general.

2.1   A Historical Perspective

The recorded study of the salmon louse dates back to the eighteenth century when Linnaeus first described a parasitic copepod, then labelled *Pediculus farionis*, found on Norwegian salmon in 1761. From the description of the parasite and the nature of the host it is believed to be the first description of a caligid and probably *Lepeophtheirus salmonis* (Heegard 1947). In 1767, Linnaeus erected the genus, *Lernaea*, which was followed by several descriptions by both Linnaeus and Müller of species designated for the genus. It was in his memoirs that Müller (1777) described another caligid copepod, *Lernaea pectoralis* (later redescribed by Nordmann as *Lepeophtheirus pectoralis*). At the time of these and other descriptions, the sum of knowledge of parasitic copepods was very limited, with the result that most descriptions were later reclassified to accommodate the increasing numbers of discovered species along with the knowledge about them. In 1785, Müller established the genus *Caligus* with a description of *Caligus curtus* that typified the caligid-type copepods, which was actually very similar to the yet to be named genus *Lepeophtheirus*. Originally Müller labelled the genus *Binoculus* referring to what he believed to be two large eyes on the frontal plates. Later, after realizing that the two large disks (known today as lunules) were not eye

spots, and failing to find eyes, he changed the name to *Caligus* from the Greek word 'caligo' which referred to blindness or weakness of the eyes (Wilson 1905). Despite the fact that *Lepeophtheirus* was yet to be established and that Müller's *Lernaea pectoralis* was later placed into it, it is interesting to note that Müller himself did not put *Lernaea pectoralis* into *Caligus*, which he established, despite the morphological similarities between *Caligus* and *Lepeophtheirus*.

It wasn't until 1832 that *Lernaea pectoralis* was moved into a new genus, that of *Lepeophtheirus*, which was proposed by Nordmann, to become known as *Lepeophtheirus pectoralis*. Although the term "louse" is commonly used to describe many crustacean ectoparasites, the term *Lepeophtheirus* signifies the first official reference as it comes from the Greek words meaning 'scab' and 'louse' (Wilson 1905).

At the time when Nordmann separated *Lepeophtheirus* from *Caligus* he described *Caligus elongatus*, which became synonymous with another Caligid, *Caligus rapax*, until the identities of the two were resolved by Parker (1969). In 1838 Krøyer described the common salmon louse *Lepeophtheirus salmonis*, originally named *Caligus salmonis*; however, it was probably known as a common fish parasite well before (Heegard 1947; Kabata 1979). Kabata (1979) gives a more complete review on the history of caligid systematics and it is interesting to note the many changes in nomenclature in later descriptions of copepods which were believed to be new species when in fact they were already known. Kabata (1979) lists 11 synonymous descriptions of copepods which are all actually *Caligus elongatus*.

2.2 Taxonomy

The parasitic copepods have been divided, from the order Copepoda, into three suborders based largely on the morphology of the mouth; these are the Poecilostomatoida, the Cyclopoida, and the Siphonostomatoida (Kabata 1979). Of these suborders the Siphonostomatoida is the largest, accounting for approximately 75% of the known species of parasitic copepods. It is in the Siphonostomatoida that we find the Caligidae, which contains 27 genera with over 350 species (Yamaguti 1963; Kabata 1988). Within the Caligidae, *Caligus* represents approximately two thirds of the total family, comprising of over 200 species, whereas *Lepeophtheirus* represents the later third with approximately 100 representatives (Yamaguti 1963; Kabata 1988). Both species are exclusively marine parasites of fishes and elasmobranchs save for one species, *Caligus lacustris*, which inhabits freshwater, and a few representatives which can be found in brackish water.

Apart from having the classical Siphonostomid-type mouth, where the labrum and labium are fused together forming a mouth tube or cone (Kabata 1974; 1979), the caligids are characterized by three main morphological features; the main body is dorsoventrally flattened with the anterior covered by a dorsal shield, dorsal or dorsal lateral plates are absent, and the cephalothorax incorporates the first three leg bearing segments (Kabata 1979). *Caligus* is easily distinguished from *Lepeophtheirus* by the presence of lunules, which are small sucker-like disks located on the anterior margin of the frontal plates.

2.3 Morphology

In general, the life cycle of caligid copepods comprises of 10 stages: 2 planktonic

naupliar stages, 1 semiplanktonic infective copepodid stage, 4 larval chalimus stages, 2 preadult stages and 1 adult stage (Kabata 1972) (Figure 2.1).

There are no complete descriptions of *Caligus elongatus* which include all stages of the life cycle; however, descriptions of the nauplius and copepodid are given by Taylor (1987) and the adult male and female by Kabata (1979; 1988). In Wilson's (1905) monograph there are descriptions of *C. rapax* which he believed to be synonymous with *C. elongatus*. A complete description of each of the developmental stages of *Lepeophtheirus salmonis* is given by Johnson and Albright (1991c). Incomplete descriptions in literature on the various developmental stages of *L. salmonis* include: nauplius (Johannessen 1978); copepodid (White 1942; Taylor 1987); chalimus 1, 2 & 4 (White 1942); adult male and female (Kabata 1979; 1988).

Apart from the obvious differences in size and the presence of lunules in the genus *Caligus*, differences in the morphology of the various species of the Caligidae are subtle and can only be ascertained via dissection and examination with a high-powered microscope. However, the general habits of all the Caligids are the same and the following is a general description of the morphology of *Lepeophtheirus salmonis* and *Caligus elongatus* adults (taken from Kabata (1979)) (see also Figure 2.2). The Caligid body comprises of four sections or tagmata: the cephalothorax, the fourth leg bearing, or free thoracic segment, the genital complex and the abdomen (Figure 2.3). The cephalothorax is a large shield-like structure resulting from the fusion of the cephalon with the first three thoracic segments. At the anterior margin, there are two frontal plates fused to the cephalothorax and delineated by a transverse suture.
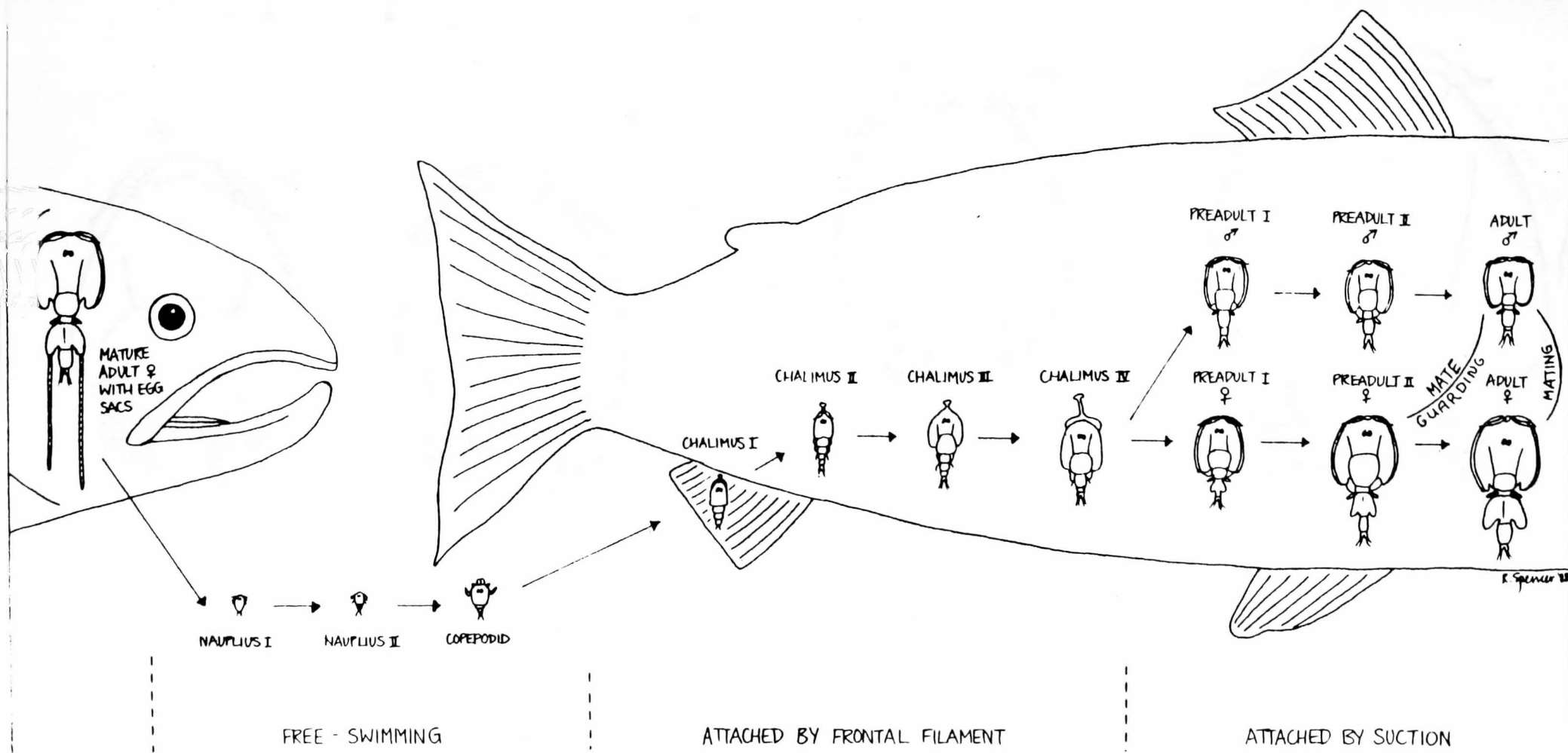
Figure 2.1. The life cycle of the salmon louse (magnification ≈ 2x) (diagram courtesy of R. Spencer).
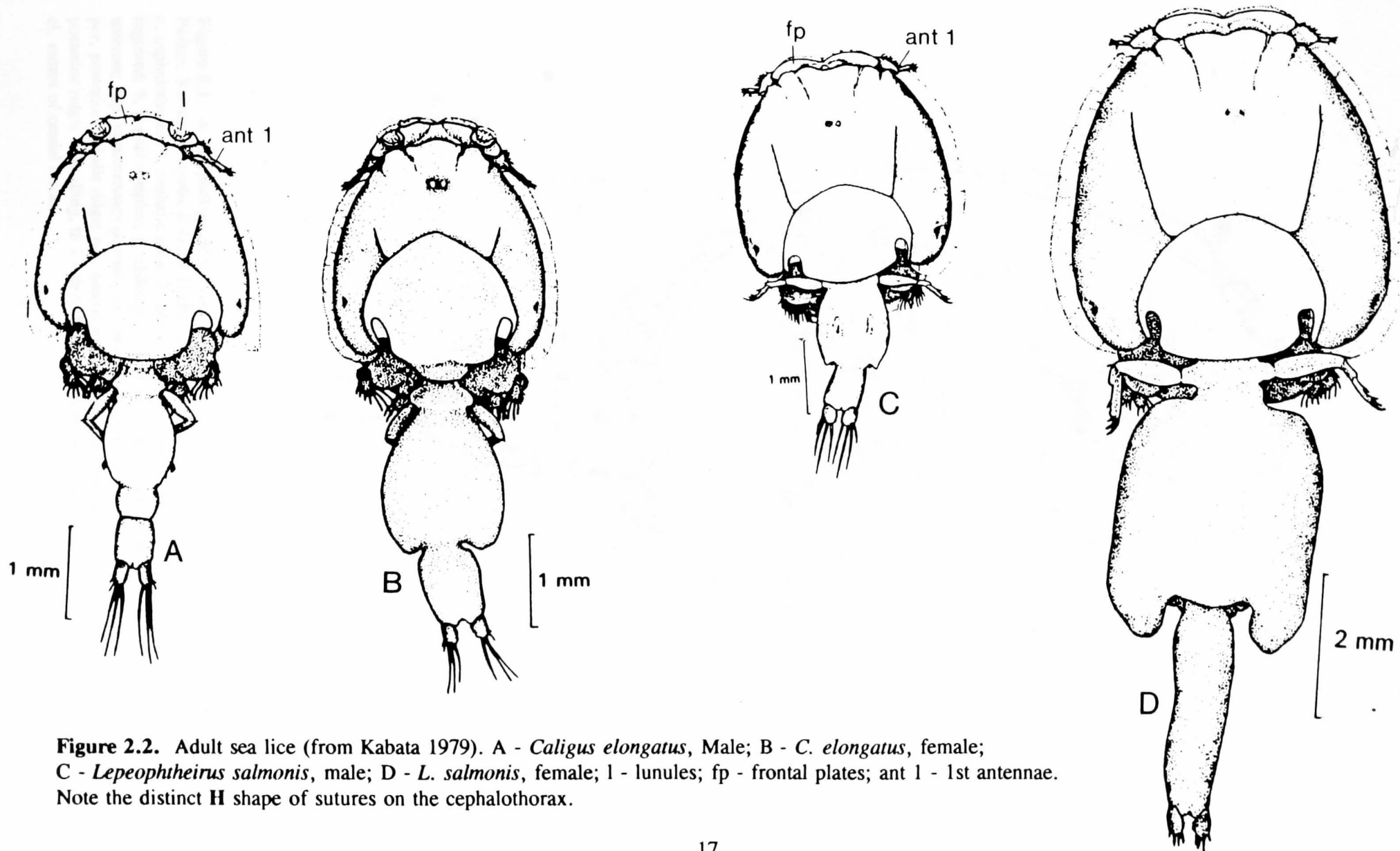
Figure 2.2. Adult sea lice (from Kabata 1979). A - *Caligus elongatus*, Male; B - *C. elongatus*, female;
C - *Lepeophtheirus salmonis*, male; D - *L. salmonis*, female; l - lunules; fp - frontal plates; ant 1 - 1st antennae.
Note the distinct **H** shape of sutures on the cephalothorax.
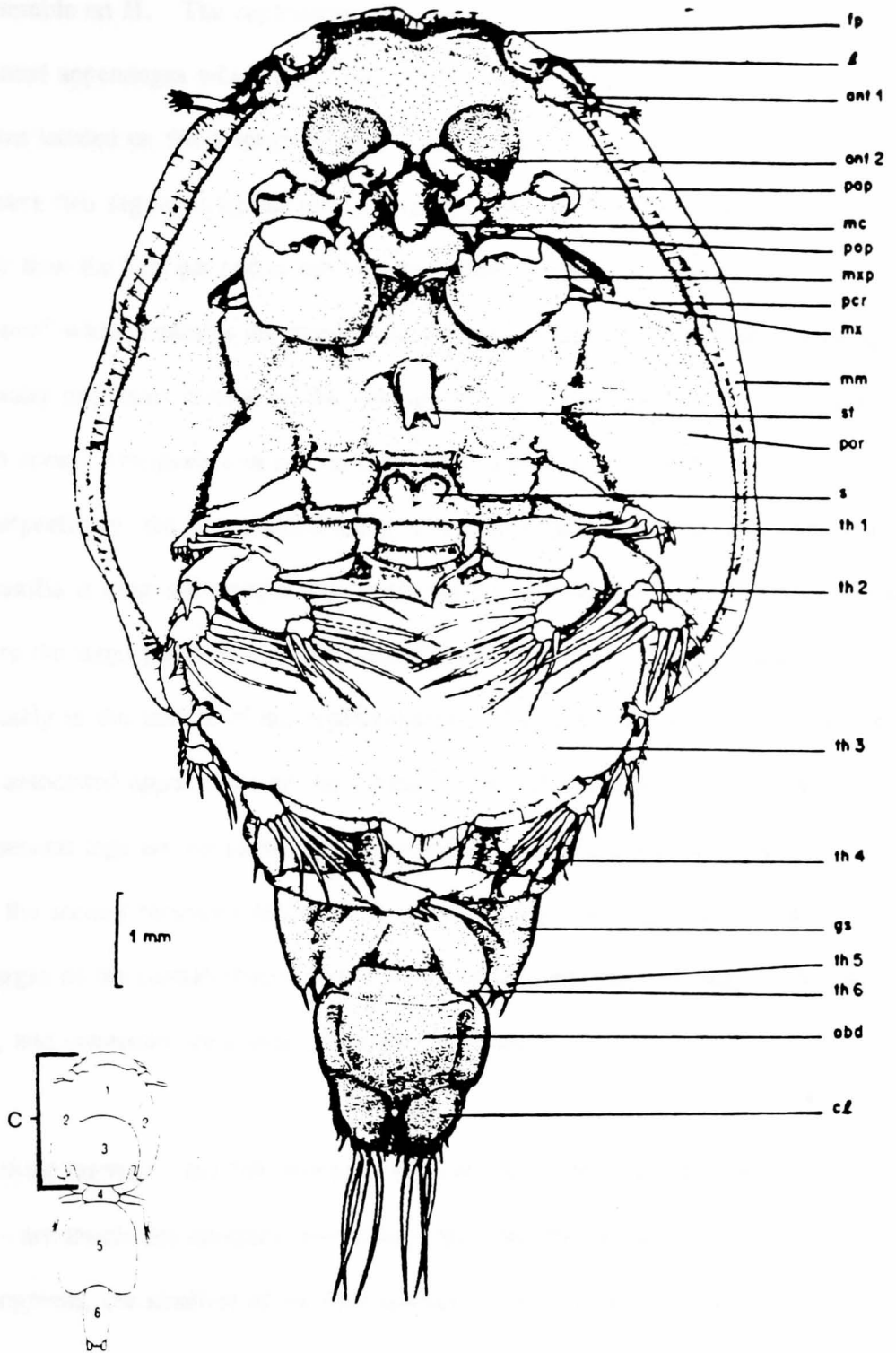
Figure 2.3. A. Morphology of the ventral surface of a adult caligid copepod (*Caligus curtus*) (from Parker, Kabata, Margolis & Dean 1968). B. Inset, dorsal view of a caligid female (from Kabata, 1979). c, cephalothorax; 1, cephalic zone; 2, lateral zone; 3, thoracic zone; 4, free thoracic (or leg bearing) segment; 5, genital complex; 6, abdomen; fp, frontal plate; l, lunule; ant 1, first antenna; ant 2, second antenna; pap, postantennary process; mc, mouth tube; pop, process of first maxilla; mxp, maxilliped; pcr, posterior cephalic ridge; mx, second maxilla; mm, marginal membrane; st, sternal furca; por, posterior ridge; s, swelling, th 1 - th 6, first to sixth thoracic legs; gs, genital segment; abd, abdomen; cl, ramus of caudal furca.

Dorsally, the areas of the first three thoracic segments are delimited by a set of sutures which resemble an H. The cephalothorax is the location of the majority of the differentiated appendages which are found on the ventral surface. There are two sets of antennae located on the anterior margin of the cephalothorax (the frontal plate); the first set have two segments tipped with numerous setae, the 2nd set is more complex and larger than the first set and is sub-chelate. Moving posteriorly, there is a short "mouth cone" which contains the mandibles. In both genera there are well-developed postantennary processes located on the ventral surface of the cephalothorax alongside the mouth cone. Alongside and posterior to the mouth cone are the first and second maxilla respectively, the first maxilla being short and tipped with a papilla, while the second maxilla is long and slender with branched tips. Posterior to the first and second maxilla are the large prehensile clawed maxillipeds. The sternal furca is located approximately in the middle of the cephalothorax. The legs are posterior to the mouth cone and associated appendages on the ventral surface of the cephalothorax. Both the first and second legs are connected by interpodal bars; the first with a vestigial endopod, the second biramous (endopod & exopod). The third leg, located on the lateral margin of the cephalothorax, fused with the sympods (the two segmented leg), is biramous, and connected by a large plate-like structure.

The next three tagmata - the free thoracic segment, the genital complex and the abdomen - are much less complex morphologically than the cephalothorax. The free thoracic segment, the smallest of the four thoracic segments, lies between the cephalothorax or the genital complex. Unlike the first three segments which are fused together to form the cephalothorax, the free thoracic is not fused to the cephalothorax or genital complex, allowing articulation between the two. The free thoracic segment

is the location of the fourth leg which is uniramous. The third tagmata is the genital complex which is comprised of three fused segments. The genital complex supports vestigial fifth (males and females) or sixth (male *Caligus elongatus*) legs and is dorsoventrally flattened; its general shape varies between males and females. In female caligids, the sixth legs form genital opercula closing off genital apertures. Lastly, the final tagma is the abdomen, which is also dorsoventrally flattened, comprising of one or more segments and varying in shape according to gender. The most anterior tip is flanked by the setiferous caudal lamellae.

Sexes are easily separated by differences in size and the shape of the genital complex. The genital complex of the females is generally the shape of an upside down **W** often with trailing uniseriate egg cases, whereas in the males it is more oval shaped. Less obvious are differences in the shape of the second antennae and maxillipeds, which in the males are usually much larger and more robust, sporting various spines, pads or rough patches.

2.4   Nervous System

In the caligids, the central nervous system is comprised of two ganglia and the paired nerves which arise from them. These ganglion are located directly above and below the oesophagus and are fused to form a junction of nerve fibres such that the structure completely surrounds the oesophagus (Wilson 1905; Scott & Scott 1913). The supraoesophageal ganglion is the smaller of the two and has three pairs of nerves which service the eye spots, the 1st and 2nd antennae and the filament gland (Wilson 1905). It is interesting to note that the nerve set which services the first antennae is relative large with a complex system of fibres innervating individual setae, which may be

indicative of a sensory function in that appendage (Laverack & Hull in press). The suboesophageal ganglion is much larger and has ten pairs of nerves which service the remaining appendages and musculature (Wilson 1905).

Very little is known of the physiological mechanisms which operate the nervous system in these crustaceans. Research on the neurotransmitters involved has shown that, of the two classes of cholinesterase, acetylcholinesterases (AChE) and butyrylcholinesterase, sea lice contain AChE, and that the specificity of the AChE is less than that found in salmon (Walday & Fonnum 1989a). AChE activity is present in all life stages including non-pigmented and pigmented eggs, while choline acetyltransferase activity can be found in all stages including pigmented but not in non-pigmented eggs (Walday & Fonnum 1989b). With both enzymes, cholinergic activity increases with each developmental stage (Walday & Fonnum 1989b). Histochemically, AChE can found to be highly localized in the paired central ganglion around the oesophagus (Walday & Fonnum 1988b).

2.5   Musculature/Locomotion

A general account of the musculature of the caligids can be found in Wilson's (1905) monograph and more recently by Boxshall (1990). The method of feeding by caligid copepods was studied by Kabata (1974), and involves rasping movements of a toothed ridge, the strigil, which is located inside the mouth cone. Tissue which is scraped off the host then moves into the mouth with the aid of a set of mandibles also located inside the mouth cone.

With respect to locomotion, caligids appear to propel themselves by antagonistic

movements of the first two thoracic legs which produces a jet when they beat together (Kabata & Hewitt 1971). On the recovery stroke the second thoracic leg, made water-tight by a thin membrane, pushes a jet of water through a sinus located between the second and third leg, thus keeping the parasite pressed against the host in addition to producing forward motion (Kabata & Hewitt 1971). When attached, movement is restricted to forward motion, as backward movement is prevented by the sternal furca and the protopodite of the third leg (Kabata & Hewitt 1971; Gnanamuthu 1948). However, it is thought that copepods may become "restless" on slow swimmers and it is at this time that they change host (Kabata & Hewitt 1971). Kabata & Hewitt (1971) reported that the best time for catching free-living caligids in the plankton was at night when fish were inactive; however, Neilson *et al.* (1987) found that free swimming *Caligus elongatus* were most abundant between 0400 and 0759 hrs (June) at one sampling site and between 0800-1159 and 2000 - 2359 hrs at another. Although adult caligids can be found in the water column (Gnanamuthu 1948; Kabata & Hewitt 1971; Neilson *et al.* 1987), the distribution of the infective copepodid stage in the plankton is unknown.

2.6 Circulation and Respiration

Circulation in the Caligids is achieved by irregular streaming of corpuscles in the spaces between various internal structures (Wilson 1905; Scott & Scott 1913; Gnanamuthu 1948). As far as is known, there are no structures similar to a heart or blood vessels. The system is driven by the contractions and dilations of the muscles which control the movement of the body, simultaneously causing body fluids to flow back and forth (Gnanamuthu 1948). Although there are no defined blood vessels there are distinct routes of flow which can be found throughout the body (Wilson

1905). There is a main current which lies underneath the alimentary canal and runs anterior to posterior; this central current then feeds two sets of lateral currents which both originate from a region just behind the eyes. The first of the two sets of lateral currents circulates in arcs in the carapace, while the second runs posteriorly, servicing the thoracic area, the free segment, and the genital segment before rejoining the central current.

Although there is no structure similar to a heart, there are two points along the median current with valve-like action (Wilson 1905). The first is located at the apex of the posterior thoracic joint where there are three valves, one central and ventrally placed and two on either side dorsally. The dorsal and ventral valves open alternatively and produce about 30-40 regular pulses per minute. There is also a single one-way flap valve which is located at the basal joint of the second antennae. Individual corpuscles tend to flow in one direction due to the series of one-way valves, with the haemolymph flowing forward in the median sinuses and backward in the lateral sinuses (Gnanamuthu 1948).

Since the majority of the exoskeleton cuticle is relatively thick, it is believed that caligids, and other copepods, respire via osmosis through the ectodermal lining of the rectum where the cuticle is thinner (Wilson 1905). The musculature which controls the opening and closing of the anus is described by Boxshall (1990). Although Hartog (1884) first proposed the notion of rectal respiration in free living copepods, Gnanamuthu (1948) did not observe the rectum taking in water during defecation or at any other time in individual caligids, thus casting a shadow of doubt on the rectal respiration hypothesis. However, it has also been speculated that the pore canals and

glands in the basal joint of the protopodite of the second and third pairs of legs may act as respiratory structures (Scott & Scott 1913). Some caligids have been shown to be tolerant to anaerobic conditions for up 9 hours. Krishnaswamy (1960) showed that following exposure to anaerobic conditions for varying lengths of time, *Caligus diaphanus*, parasitic on gurnards, required 2-4 min to recover from 3 hours exposure, 4-15 min to recover from 4 hours exposure and 20-30 minutes to recover from 8-9 hours exposure.

2.7 Osmoregulation

Despite a great deal of literature on marine parasitic copepods, very little is known of the mechanisms which regulate their basic physiological processes such as osmolarity. In general, caligids parasitizing wild salmon die when the salmon run up fresh water river systems to spawn. Salmon lice have been known to remain attached to running salmon for periods of a week (Hutton 1923) or up to twenty five days (Ashby 1951). More recently, McLean, Smith & Wilson (1990) showed that on average most lice (93%) are lost within 48 h of immersion in freshwater, with some animals surviving for 5, and in one instance 6, days. It is possible that the relatively colder temperatures in the early studies (7.5 - 8.0°C) are related to the longer survival times than reported by McLean *et al.* (1990) who conducted their studies at temperatures ranging from 12.8 to 16.0°C.

In the laboratory, the lower tolerance limit of sea-lice to salinity is 16‰ (Berger 1970); however, it is interesting to note that Berger (1970) found that the nauplius were more tolerant to low saline conditions than adults. However, Johnson & Albright (1991a) reported that no nauplii developed from eggs incubated at a salinity of 15‰

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despite a 70% hatch rate. In these trials Johnson & Albright (1991a) reported that optimum hatching of nauplii occurred at salinities of 25 and 30‰; however, active copepodids could only be reared at 30‰. In general, the survival of newly moulted copepodids is significantly higher at 30‰ than 15‰ for a range of temperatures (5, 10 & 15°C) (Johnson & Albright 1991a). Optimum temperature and salinity for copepodid survival were reported to be 10°C and 25‰ (Johnson & Albright 1991a). Johnson & Albright (1991a) also reported survival times of adult lice at different salinities, *in vitro*, and found marginally lower survival times at 10 & 15‰ (12- 13 days) than at 30-31‰ (18 days), suggesting a certain level of tolerance to low saline conditions. The upper limit for salinity tolerance is unknown; however Gnanamuthu (1948) reported that a salinity of 43‰ (artificially prepared) was toxic to adult *Caligus savala*.

It is believed that cell volume regulation is mediated, in part, by a set of osmoeffectors which include glycine, alanine, proline and taurine (Hahnenkamp & Fyhn 1984). As tissues become diluted, these osmoeffectors enter the cell to adjust intracellular osmolarity and can be found in a wide variety of invertebrates and vertebrates (Gilles 1979). Briggs (1977) believed that various gut cells in *Paranthessius anemoniae* (a parasitic copepod of sea anemones) which were high in mitochondria and smooth endoplasmic reticulum may also play a role in osmoregulation. In addition, defecation may be a behavioral response which may be osmoregulatory and/or ionoregulatory (Gnanamuthu 1948; Fox 1952).

Sea lice alter their behaviour in response to different osmotic conditions. In 37% sea water, Hahnenkamp and Fyhn (1984) found that, whatever mechanism was involved in osmoregulation, it was independent of the host, as no difference could be found

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between attached and unattached adult lice. In fresh water, however, the host plays a major role in the ability of the lice to deal with the sudden osmotic shock, with attached lice surviving for a week opposed to free swimming lice surviving eight hours (Hahnenkamp & Fyhn 1984). There are two mechanisms believed to be responsible for survival in low salinities; the first simply involves the feeding by the lice, which replaces lost body salts (Hahnenkamp & Fyhn 1984), while the second mechanism, demonstrated by the slower rate of change in haemolymph osmolality and Cl^- concentration, is believed to be the result of the louse pressing deeper into the skin of the host, which helps to reduce diffusion (Hahnenkamp & Fyhn 1984). A similar effect of host dependence was seen in *Lernaea cyprinacea* (Shields & Sperber 1974). Ultimately, death from osmotic shock is believed to be caused by cell volume regulation mechanisms (Hahnenkamp & Fyhn 1984). Whether or not the cuticle plays a fundamental role in osmoregulation is unknown. Similarly, in the case of caligids, it is not know whether or not there is a difference in the osmotic tolerance between attached chalimus stages and the adult stages.

2.8 Feeding

As mentioned earlier, a characteristic feature of caligids is the Siphonostomid-type mouth, or mouth tube or cone. The Siphonostomid mouth tube is the generally the longest found in the parasitic copepods and possesses a serrated ridge known as the strigil (Kabata 1974). However, within the Siphonostomids, the mouth tube of the caligids is comparatively short. Having looked at the structure of the caligid mouth in detail, Kabata (1974) theorized on the mechanics involved in feeding; to feed, the mouth tube is pushed into the host tissue and in doing so pushes back the labial fold and exposes the strigil. The strigil, which lies on the horizontal plane of the mouth

opening, is a divided bar connected in a way that allow the two halves to pivot about a common base. As the mouth is pushed forward and back by the copepod, the two halves of the strigil swing back and forth, lacerating the tissue below it. Mandibles inside the mouth tube manoeuvre food particles into the mouth where peristaltic action takes it to the oesophagus. Boxshall (1990) gives a detailed description of the musculature associated with the caligid mouth tube.

The first two free living stages of the caligid life cycle, the nauplius 1 and 2, do not possess any feeding appendages or anus and during naupliar development do not feed (Johannessen 1978). The third stage, the copepodid, is the first stage in which the mouth cone appears and the stage where active feeding begins.

The caligid gut is rudimentary, comprising an oesophagus, a stomach which is continuous with the intestine, a short rectum and finally the anus which terminates at the posterior of the abdomen (Scott & Scott 1913). The oesophagus runs through the supra-oesophageal ganglion and forms a very narrow tube which opens up into the wider anterior stomach. At the anterior end of the stomach a caecum extends dorsally over the posterior end of the oesophagus. There are no prominent convolutions in the gut and the wall of the entire tract is lined with a thin layer of chitin. Along the length of the gut in the genital segment and abdomen, adults and chalimus possess small oil droplets which are believed to be food storage sites (Gnanamuthu 1948). As was mentioned earlier, food is conveyed along the gut via peristaltic contractions. These contractions, which travel up and down the gut, occur at irregular intervals and originate from four focal centres along the gut (Gnanamuthu 1948).

In addition to the above, Scott and Scott (1913) noted the presence of two sets of digestive glands. The larger of the two comprise of three sections located at the anterior end of the cephalothorax. Two larger sections can be found just behind the antennules and are connected via a duct to the smaller third section, located in front of the base of the mouth. A duct from this smaller section runs posteriorly along the oesophagus and opens into the caecum. The second set of glands, described as being somewhat "comma shaped", are located medianly between the first and second thoracic legs. Ducts from the glands pass along the stomach and enter it near the posterior end. The purpose of these glands is unknown, but they are believed to secrete substances which aid in digestion (Scott & Scott 1913).

Since sea lice are found on the body of fish hosts, many researchers concluded that lice feed upon mucus and epidermis (Scott & Scott 1913; White 1942). Other authors suggested that, in addition to mucus, host skin and subcutaneous tissues, a red pigment seen in the gut indicated the possible ingestion of blood (Voth 1972). Scott & Scott (1913) speculated that the digestive glands may play a role in altering ingested food, thereby masking its origin. Using spectrophotometric techniques Brandal, Egidius & Romslo (1976) showed that the fluid found in the gut of adult females and males as well as in post chalimus stages of both sexes was salmon blood. Of the two, C. elongatus and L salmonis, C. elongatus has a wider array of proteolytic enzymes for digestion, possibly reflecting some mechanism to deal with a wider range of hosts (Ellis, Masson & Munro 1990). In the absence of a host, lice have been maintained in aquaria for up to six weeks (Scott & Scott 1913). Johannessen (1978) kept a tank-reared copepod alive for 30 days in the absence of food.

2.9   Reproduction

The organization of the reproductive system is uniform throughout caligids, and as such the following discussion will not only apply to *Lepeophtheirus salmonis* and *Caligus elongatus* but to all the members of the family. An excellent discussion on the anatomy of the reproductive organs can be found in Wilson (1905) and in Scott & Scott (1913). The following is a brief summary taken from Wilson (1905) describing basic anatomy.

The main components of the reproductive system in the females consists of the ovaries, oviducts, seminal receptacles and the cement glands. The characteristic kidney-shaped ovaries are located in the cephalothorax anterior to the stomach. From the ovaries the oviduct emerges as a thin tube which runs in a straight line posteriorly through the thoracic area and free segment and into the genital complex where it becomes greatly enlarged and convoluted. Near to the terminus of the oviduct on the ventral side is the cement gland, which is an oblong transparent organ that produces the egg case material, which both surrounds and separates the eggs. Both the shape of the cement gland and the arrangement of the convolutions are believed to be species specific (Wilson 1905). Egg strings are extruded via a set of pores, the os uteri, located on the posteriorly on the ventral surface of the genital complex.

The seminal receptacle is a horseshoe-shaped organ which is enlarged at the distal ends. It extends from both of the os uteri and is connected to the spermatophores via a narrow tube, the vagina, and to another set of external pores, the vulva, which are located on the posterior margin of the genital complex.

Female *L. salmonis* can carry between 200 and 1400 eggs, with a mean of 107-315 per

egg string (Johannessen 1978; Wootten *et al.* 1982; Kunz 1985; Tully 1989). The

average fecundity of *C. elongatus* is 100 eggs per string with upper and lower limits at

30 and 180 respectively (Hogans & Trudeau 1989a; Taylor 1987; Kunz 1985). In most

instances variation in fecundity of both species was found to be high (Kunz 1985;

Taylor 1987). The number of eggs laid appears to be unrelated to environmental

factors such as temperature in *C. elongatus* (Hogans & Trudeau 1989a; Tully 1989).

However, *L. salmonis* has been known to produce three times as many eggs in January

as in August (Tully 1989). In terms of incubation times, Johannessen (1978) found that

the egg-bearing period, for *L. salmonis*, is inversely related to temperature such that at

9°C the eggs would hatch, *in vitro*, in 31-39 days and at 11.5°C eggs would hatch in

10-13 days. In contrast, Johnson & Albright (1991a) reported egg developmental times

of 5.5, 8.6 & 17.5 days at 15, 10 & 5°C respectively. Optimum temperatures for

hatching were found to be between 5 and 22°C (Johannessen 1978); however, lower

than optimal temperatures during embryonic development had a detrimental effect on

further development of the eggs and usually ended in aborted eggs (Johannessen 1978).

There is no data on the incubation times of *C. elongatus*.

The onset of hatching is marked by dark pigmentation in the eggs (Johannessen 1978) a

feature found in many of the Siphonostomids (Heegard 1947; Roth 1988). The

mechanisms involved in the hatching process are poorly understood. Heegard (1947)

believed that eggs moved from within the egg case to the exterior when driven by

osmotic forces. However, close observation of the nauplius just prior to hatching

reveals a great deal of activity which, via mechanical means, may move individual eggs

to the exterior of the egg case and allow the nauplius to break free (Lewis 1963; Roth

1988). Whichever the means, once the hatching sequence begins, hatching progresses from the distal end of the egg string to the anterior (Johannessen 1978; Johnson & Albright 1991a). If, however, eggs are observed hatching *in vitro*, eggs hatch simultaneously at many locations along the egg string (Kunz 1985).

In the male the reproductive organs consist of the testes, vas deferens, seminal vesicle, and the cement gland. The location of the reproductive organs is the same as in the female with obvious differences in function. The testes are found in the same location as are the ovaries in females but are considerably smaller. From the testes the vas deferens leads straight down into the genital complex to the oval shaped seminal vesicle. The cement glands are situated on the ventral side of the seminal vesicle towards the outside margin. The cement gland produces a sticky viscous substance which aids in spermatophore attachment. Sperm is collected in the seminal vesicle and shaped into an oval spermatophore which is deposited onto the female during copulation. Spermatogenesis is discussed, in brief, for *Pandarus sinuatus* (an allied siphonostomid parasitic of sharks) by McClendon (1906).

Wootten *et al.* (1982) reported that adult males preferentially mate with preadult 2 females but will also mate with adult females. However, Anstensrud (1990b) reported that although males take precopula positions with preadult females in *Lepeophtheirus pectoralis* (parasitic on flounder), they only copulate once the female has moulted to the adult stage. In one instance copulation was observed between an adult male and a preadult female, but the resultant eggs where not viable (Anstensrud 1990b). It is thought that a single set of spermatophores contains enough sperm to fertilize all the eggs produced in one female's lifetime, which suggests that copulation may only occur

once. Anstensrud (1990b) reported that, for *L. pectoralis*, 54% of a laboratory population were inseminated once and 46% of the population was inseminated twice with no individual females being inseminated a third time. Unfertilized females tend to have a smaller genital complex (Scott & Scott 1913; Anstensrud 1990c) suggesting that mating is required for maturation. Fertilization as a maturation cue has also been reported in other, allied, parasitic copepods (e.g. *Lernaea cyprinacea, Lernaeocera branchialis*), (Bird 1968; Anstensrud 1990c).

The mating behaviour of *Lepeophtheirus pectoralis* was studied in detail by Anstensrud (1990b). Prior to copulation, adult males took up precopula positions on preadult females, by grabbing onto the genital complex dorsally. However, when the female moulted, males would temporarily leave females and return shortly after the moult was complete. There was no competition observed between males without female mates and those with mates, however; males sometimes lost partners during the short moult interval to satellite males in the vicinity of the pair. Following completion of the final moult, males then moved to the copula position by moving around to the ventral side of the genital complex. During copulation the male grasps the female's genital complex with the second antenna and maxillipeds and then transfers a set of spermatophores with its second swimming legs to the vulva of the female. The spermatophores are attached to the female by a sticky cement-like substance secreted by the male.

Eggs arising in the ovaries move along the oviducts and take shape along the way. Fertilization takes place when they pass the seminal receptacle and are deposited into the egg strings. Sperm moves into the female seminal receptacle via a tube like structure associated with the spermatophore, and therefore produced by the male

(Anstensrud 1990b). Once deposited, the tube everts from the spermatophore, and then extends into the female seminal receptacle (Anstensrud 1990b). Interestingly, the cement which functions to attach the spermatophore also actively seals the vulva leading to the female seminal receptacle (Anstensrud, 1990a). This latter observation is interesting in light of the early findings by Anstensrud (1990b) on the lack of multiple copulations in *Lepeophtheirus pectoralis*. The process of egg laying in *Caligus spp.* is briefly summarized by Heegaard (1959). Aspects of embryology, for *Pandarus sinuatus*, are discussed by McClendon (1906; 1907).

2.10   The Caligid Life Cycle

The caligid life-cycle comprises 10 stages: two planktonic naupliar stages, 1 semi-planktonic infective copepodid stage, 4 attached chalimus stages, 2 unattached parasitic preadult stages and 1 unattached parasitic free living adult stage (Kabata, 1972). The term "metanauplius" was used in previous descriptions of caligids, to describe the second nauplius (Hwa 1965) and the copepodid (White 1942); however, this terminology has not gained general acceptance and is not used. To date, nine caligid life cycles have been described in full, including *Caligus centrodonti* (Gurney 1934), *C. curtus* (Heegard 1947), *Lepeophtheirus dissimulatus* (Lewis 1963), *C. orientalis* (Hwa 1965), *C. spinosus* (Izawa 1969), *C. clemensi* (Kabata 1972), *L. hospitalis* (Voth 1972), *L. pectoralis* (Boxshall 1974a) and *L. salmonis* (Johnson & Albright 1991c). It should be pointed out that, of these nine life cycle interpretations, only three recorded ten individual stages. Although Kabata (1972) suggested that the 10-stage life cycle is characteristic of all caligids, he also reported difficulties in finding the second preadult in laboratory cultures of *C. clemensi*, indicating the difficulty in delineating between the different life stages.

In *L. salmonis* the first naupliar stage lasts approximately 12 to 35 h at 15.5 to 9.2°C respectively (Johannessen 1978). Similar developmental times of 9.2, 30.5 and 52 hours at 15, 10 and 5°C were reported by Johnson & Albright (1991a) and 15 to 30 h (temperature not given) by Hogans & Trudeau (1989b). As stated earlier, the nauplius does not feed, and, being positively phototactic, migrates vertically to the surface layers of the water column (Johannessen 1978; Hogans & Trudeau 1989b). The second nauplius stage of *L. salmonis* lasts approximately 42 h at 9.2°C; however, only one individual was observed by Johannessen (1978). In contrast, Johnson & Albright (1991a) give a somewhat shorter estimate of 31.2 h (10°C). In *C. elongatus* the second nauplius stage lasts 35 h at 10°C (Hogans & Trudeau 1989b). Hogans & Trudeau (1989b) observed no moulting in *C. elongatus* at temperatures below 3°C. It is believed that positive phototaxis serves as a mechanism to bring the nauplii up the surface where it is most likely to encounter hosts (Hogans & Trudeau 1989b). Although the nauplius has no method of attachment it has been observed to burrow into the mucus of salmon (Hogans & Trudeau 1989b).

Unfortunately, Johannessen (1978) only observed 2 copepodids in his *in vitro* trial; with one surviving 2.6 d at 11°C and the other surviving 1.4 d at 19°C. Johnson & Albright (1991a) reported that copepodids could survive for up to 10 d at 10°C, while Hogans & Trudeau (1989b) reported that the *C. elongatus* copepodid stage lasts 50 h at 13°C. Since developmental time is heavily dependant on temperature it is difficult to compare developmental times between various other caligid species. In *C. elongatus* the copepodid stage lasts 50 h at 13°C (Hogans & Trudeau 1989b). Similar to the nauplius, copepodids are positively phototactic, and within distances of approximately 10cm are reported to detect chemotactic cues (Hogans & Trudeau 1989b).

Once a host is located, the copepodid attaches with prehensile second antennae (Bron, Sommerville, Jones & Rae 1991). The parasite then begins secreting the frontal filament which anchors it permanently to the host, after which it begins moulting through the chalimus series (Bron *et al.* 1991). In some instances adult gravid females have been observed whilst attached by a frontal filament (Hogans & Trudeau 1989a). A similar observation was made by Anstensrud (1990b) who concluded that preadult and adult *L. pectoralis* secrete a temporary filament during moulting. Experiments on the settling behaviour of copepodids indicated a slight preference for the ventral fins of healthy fish over fish in poor condition (as measured by Fulton's K, = "condition factor" (weight/length^3)) (Taylor 1987). Neilson *et al.* (1987) found no correlation between condition factor and parasite load in cod and haddock; however, smaller fish tended to carry higher parasite burdens. Bron *et al.* (1991) also reported a preference for settlement on the fins; similarly Herrera (1990) reported that *Lepeophtheirus* chalimus (species not given) parasitizing wild anchovy larvae (*Engraulis ringens*) were always found at the base of the pectoral fins. In some instances, laboratory studies have shown that settlement of *L. salmonis* copepodids may occur on the gills or in the buccal cavity (Bron *et al.* 1991; Johnson & Albright 1991a), however, since there are no similar reports in wild fish, these settlement sites may represent laboratory induced artifacts.

It is interesting to note that none of the researchers who examined growth *in vitro* observed the development of a frontal filament and the progression to the chalimus one stage. In many instances copepodids could be induced to settle onto artificial substrates such as cheese cloth or fine nylon mesh or explanted fish tissue, via second antennae, unfortunately after attachment subsequent development stopped (Lewis 1963;

Johannessen 1978; Jones 1989). Jones (1989) reported limited success in incubating explanted fish tissues with various larval stages attached (including the copepodid) with moulting observed in individual cases; however, no second moults were observed and the one preadult (from a chalimus 4) obtained in the study died shortly after moulting.

The development of *L. salmonis* chalimus stages was reported by Johnson & Albright (1991a) who reported developmental times of 5, 5, 9 & 6 d for Chalimus 1 through 4 respectively, with the first and last chalimus stage appearing 29 and 35 d from the beginning of the experiment (therefore including egg developmental time). Preadult males began to appear after 32 d, with the preadult 1 stage lasting 8 d and the preadult 2 lasting 10 d, whereas preadult females began to appear after 36 d, with the preadult 1 stage lasting 9 d and the preadult 2 stage lasting 12 d. Adult males appeared on day 40, and adult females appeared on day 52. Developmental time for the four chalimus stages of *C. elongatus* is 21 d at 12°C (Hogans & Trudeau 1989b). There are no reports on the developmental times of the adult stages of *C. elongatus*.

Populations of *L. salmonis* cycle throughout the year, with highest numbers occurring in the late summer and mid fall (Hogans & Trudeau 1989b; Tully 1989; Wootten *et al.* 1982). The pattern begins in May with the majority of the population comprised of gravid females, followed by successive generations of chalimus, preadults and adults. It has been estimated that the generation time is 48 d at 16°C and 92 d at 7°C (Tully 1989). Wootten *et al.* (1982) interpolated the generation time to be 42 d at 9-12°C. A similar estimate of 52.5 - 56 d (10°C) was reported by Johnson & Albright (1991a) following direct observations of a laboratory reared population. It should be noted that in his study, Tully (1989) observed a marked lack of development and maturation,

which may have been the result of some unknown cause that subsequently resulted in longer development times. Tully (1989) believed that *L. salmonis* were failing to mature due to high temperatures. Conversely, Hogans & Trudeau (1989b) postulated that the phenomenon of low numbers of parasites occurring during the coldest months was possibly due to an inability to overwinter implying that the parasites require higher temperatures to develop. With the generation times given above, the average parasite population will cycle three or four times between May and October depending on the temperature. *L. pectoralis*, shows a similar cycling pattern but with a distinct alternation of generations (Boxshall 1974a). *C. minimus* also shows a distinct annual variation (Paperna 1980). In both cases, population parameters were believed to be temperature dependant.

Overwintering is believed to occur in *L. pectoralis*, as only adult females can be found on the host in the spring (Boxshall 1974a). In June collections of *L. salmonis* from wild fish, White (1940) found only females with pigmented egg strings and no immature copepods, and thus concluded that the females overwintered. However, on farmed salmon in Scotland all life-cycle stages are found throughout the winter. Apart from the obvious role that temperature plays on development, optimum temperatures for growth vary between species. However, rapid changes of temperature produce signs of shock in sea lice, and rapid increases (i.e. 16°C to 30°C) can lead to death (Gnanamuthu 1948). Hogans & Trudeau (1989b) speculated that the parasites did not overwinter and the increased abundance in the spring was due to immigration of parasites from wild populations.

Generation times for *C. elongatus* are somewhat shorter; 49 d at 16°C; 81 d at 8°C

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(Tully 1989) and 35 d (laboratory data) at 10°C (Hogans & Trudeau 1989b). Tully (1989) found a marked succession of generations, 3 in a six month sampling period, and noted the parasite intensities were not cumulative with generation as predicted by Wootten *et al.* (1982). In addition, Tully (1989) suggested that transfer of parasites from wild fish was not a significant factor in population dynamics as suggested by Wootten *et al.* (1982). As with *L. salmonis*, maturation failure has been recorded in *C. elongatus* (Wootten *et al.* 1982). In one instance, a high proportion of larval stages in late October/early November did not show up in later samples as adult stages. Wootten *et al.* (1982) believed that this anomaly was either due to host switching or failure of the larval stages to develop, which may indicate a degree of overwintering occurring in *C. elongatus*.

As with many parasite population structures, sea-lice are most commonly found overdispersed. With the higher fecundity and longer generation times in colder weather seen in *L. salmonis*, infections should, hypothetically, come in distinct surges (Tully 1989). However, most studies on the distribution of sea-lice only cover a portion of a year, making distribution comparisons difficult.

2.11 Prevalence and Intensity Parameters

In general, caligids, depending on the particular host-parasite system, tend to be present all year round with distributions directly related to temperature (Boxshall 1974a; Paperna 1980; Hogans & Trudeau 1989b; Tully 1989). *L. salmonis* commonly occurs on salmonids of the genera *Salmo, Oncorhynchus* and *Salvelinus* in northern latitudes (Kabata 1979; 1988). The parasite has also been recorded on non-salmonid hosts; however, such host records are believed to be the exception rather than the rule

(Kabata 1979; 1988). Infestations of *L. salmonis* on wild fish have been documented (White 1940; Templeman 1967; Panasenko *et al.* 1986; Nagasawa 1987) and it is believed that wild salmon act as reservoirs for infections in farmed salmon (Wootten *et al.* 1982). Apart from temperature, intensity of infection (in wild lice populations) is related to factors such as size and age of the host (Nagasawa 1985; 1987). It is interesting to note that in his study of Pacific salmon parasite population parameters, Nagasawa (1987) found highest prevalence and intensity in chinook salmon, yet 90% of the parasites examined came from pink and sockeye salmon which represented 70% of the hosts examined. Thus, although pink and sockeye salmon exhibit relatively low infection rates they represent a more significant reservoir of parasites.

Conversely, *C. elongatus* is much more cosmopolitan than *L. salmonis* in distribution, with recorded hosts occurring throughout 17 orders of both teleosts and elasmobranchs and in both tropical and temperate climates (Kabata 1979). Because of its widespread distribution among so many hosts, it is difficult to estimate prevalence patterns in host populations (Wootten *et al.* 1982; Hogans & Trudeau 1989b). As with *L. salmonis*, wild fish (saithe, herring, mackerel) living in close association with net pens are believed to be a significant source of infection for farmed salmon (Wootten *et al.* 1982; MacKenzie & Morrison 1989; Bruno & Stone 1990). Not only are *C. elongatus* commonly found on wild cod and haddock, but significant numbers can be found free swimming in the plankton, supporting the notion of host transfer (Neilson *et al.* 1987). In the plankton, significantly more *C. elongatus* are found in mixed (as opposed to stratified) waters, with a positive correlation with depth at stratified sites (Neilson *et al.* 1987).

Of the two hosts, cod and haddock, *C. elongatus* chalimus showed definite differential preference for attachment sites, with cod carrying a heavier burden. Whether or not *C. elongatus* is a truly opportunistic parasite or an accidental parasite of salmon is unknown, as records of infections on wild salmon are rare but do occur (Boxshall 1974b). It was suggested by Wootten *et al.* (1982) that *C. elongatus*, after developing through successive chalimus stages on a salmonid host, may leave to seek another host once it has developed into a preadult. However, during certain seasons in the year, farm reared salmon can show 100% prevalence, suggesting that salmon make desirable hosts for the pests (Hogans & Trudeau 1989b).

2.12 Pathology of Sea Lice Infections

In wild populations, damage by lice is minimal with relatively few reports of severe pathology (White 1940; Panasenko *et al.* 1986). However, in intensive rearing situations conditions are optimal for the proliferation of large numbers of lice which can cause severe economic losses (Stuart 1990). In general, parasitic copepods can have two types of effects on their hosts; general and local (Kabata 1984). Local effects include those which are caused by attachment and feeding, and in some instances reproduction (Smith 1975). General effects are of a more systemic nature, resulting in general disability of the host. Acute pathology associated with sea lice infections has been examined by Boxshall (1977), Radhakrishnan & Nair (1981; 1983), Reyes & Bravo (1983) and Jones, Sommerville & Bron (1990).

Jones *et al.* (1990) identified four areas of interaction with respect to damage caused by attached chalimus of *L. salmonis*; these were second antennal attachment, filament attachment, maxilliped activity and feeding activity. Although adult stages can cause

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such considerable damage that feeding activity can expose the brain (Kabata, 1970), the chalimus stages rarely breach the basement membrane and usually elicit very little host reaction (Jones *et al.* 1990). In contrast, Boxshall (1977) reported that the persistent feeding of chalimus stages of *L. pectoralis* on *Platichthys flesus* often extended into the dermis and exposed the fin rays. When larval fish are parasitized by chalimus the damage is proportionally greater and can have a significant impact on wild fish (Neilson *et al.* 1987). A response to the filament occurs when the chalimus moults to the preadult stage and takes a free living existence. The filament left from this moult is quickly encapsulated by connective tissue and is attacked by macrophages, leaving a white nodular lesion approximately 5 cm in diameter (Jones *et al.* 1990). The nodular lesions left by the chalimus stages have either a depressed or raised white core surrounded by pigmented tissue. Focal points of necrosis occur in association with redundant chalimus filaments but the cause is unknown (Jones *et al.* 1990).

White nodules, remnants or chalimus infection, were first described by and diagnosed as a condition known as "white spot" (M'Gonigle 1931 cited in White 1940). White (1940) believed that the white spots described by M'Gonigle were characteristic of sea lice infections. More recently, white spots in salmon have also been described as a symptomatic feature of the earlier onset of sea lice infections which eventually lead to open sores (Egidius 1985). White nodules can also be the result of adult stages of caligids feeding on hosts, particularly when feeding is restricted to one area (Radhakrishnan & Nair 1983).

There are three areas of interaction between host and parasite in adult parasite pathology; these include the second antennae, the maxillipeds and the mouth tube

(Boxshall 1977). Although the "sucker like" action of the cephalothorax acts as a major component in the attachment of sea lice, there are no pathological effects associated with this type of interaction. Damage caused by the second antennae and maxillipeds is usually superficial mechanical disruption, but in cases where parasites remain in one place, damage can extend down into the dermis (Boxshall 1977). The most profound effect of sea lice infections is due to the feeding action of the adults. In wild fish populations the feeding action of sea lice causes minimal damage, but in conditions where the number of parasites becomes large, feeding becomes restricted to particular areas due to crowding (Kabata 1984). Gross signs of feeding on salmonids infected with *L. salmonis* or *C. elongatus* include grey patches on the back of the head and sub-epidermal haemorrhages in the perianal region and directly behind the dorsal fin (Egidius 1985; Wootten *et al.* 1982). Although external digestion is known in other parasitic crustaceans (e.g. *Argulus* and *Ergasilus*) (Kabata 1970), it has not been shown in the caligids.

Histologically, there is a hyperplastic response within the epidermis and intense fibroplasia and infiltration by macrophages and lymphocytes in the dermis (Smith 1975; Boxshall 1977; Radhakrishnan & Nair 1981), with large increases in the number of mucous cells in the epidermis (Kabata 1970). Loss of fluids in the epithelium leaves large spaces between cells, and connective tissue begins to slough off (Kabata 1984). Where feeding of parasites is intense, haemorrhaging occurs (Boxshall 1977; Wootten *et al.* 1982). As a result of fibrous tissue production a fibrous granulated swelling occurs (Boxshall 1977; Radhakrishnan & Nair 1981; 1983). Death in the host is believed to be ultimately the result of osmotic failure (Wootten *et al.* 1982; Neilson *et al.* 1987).

Open wounds caused by the feeding of the parasites are believed to act as portals for opportunistic bacteria such as *Vibrio*, *Aeromonas* or *Mxyobacteria* (Egidius 1985). In fresh water environments wounds can become colonized by fungal infections (Håstein & Bergsjø 1976). Although outbreaks of *Vibrio* are known to occur during sea lice outbreaks (Wootten *et al.* 1982), it is not know whether such infections are purely opportunistic or directly linked to the copepod, with the copepod acting as a mechanical vectors. The possibility that a marine parasite could act as a vector should not be ruled out (Cone & Odense 1984; Cone & Cusack 1986), though Kunz (1985) could only isolate one colony of *V. anguillarum* from bacteriological preparations taken from copepod carapaces.

3   Control of Sea-Lice in Aquaculture

Controlling sea lice is very difficult since, as with many other disease-causing organisms, the parasites are part of the natural ectoparasitic fauna.   In response to the ever growing problem of sea lice in salmon grow out sites, various measures of control have been used; some successful, some not so successful, while others are experimental.   Measures of controlling sea lice can be classified into four basic categories: management, chemotherapeutants, biological controls and others (such as the use of vaccines).

3.1   Management Techniques

Due to the design of grow-out sites for intensive salmon culture, management techniques are very difficult to employ.   Cage sites are fixed in place and the control of environmental variables such as temperature and photoperiod is, from a practical standpoint, impossible.   Since the parasite is particularly vulnerable to low salinities, a fresh water flush could be used as an effective control, and in pump ashore facilities this might be feasible.   However, in most instances, sea lice are generally not problematic at such facilities possibly due to the deep water intake plumbing.   In one instance, parasitic isopod larva, *Paragnathia sp.*, were effectively controlled by temporarily changing the water supply to tanks stocked with eels (*Anguilla anguilla*) from seawater to fresh water (Mugridge & Stallybrass 1983).   Alternatively, filtration has been shown to effectively remove plankton born parasites from land based fish farm water supplies (Litved & Hansen 1990).

At sea cage grow-out sites, fresh water "lenses", where fresh water is pumped into

cages to form a layer of fresh water over the seawater for a short period of time, have been attempted, but the fish tend to go below the lens (Stuart 1989). With *Salmincola salmoneus* (another siphonostomid), infections can be controlled by lowering water temperatures and manually removing the parasites from anaesthetized fish (McGladdery & Johnston 1988). At lower temperatures, developmental times of *S. salmoneus* were increased from 4 - 6 weeks (12°C) to 3 months (1-4°C), and the infective copepodids were incapacitated (McGladdery & Johnston 1988). With respect to sea lice infections, both light traps and covers (to reduce net pen illumination) have been tried in experimental situations to attract/distract plankton borne stages (nauplii, copepodids or free swimming adults) but methods were found to be ineffective (Taylor 1987; Huse, Bjordal, Fernö & Furevik 1990). Lastly, attempts to physically divert plankton borne stages by surrounding net pens with skirts was also found to be ineffective (Stuart 1989).

Most recently the use of year class management and fallowing has been evaluated as a means to reduce the overall lice burden in the vicinity of salmon cages (Anon. 1992). Overall lice burdens are found to be reduced where first and second sea winter fish are separated, presumably due to the susceptibility of the younger fish. In the above mentioned study, lice numbers were found to be negligible for a period of 7 months following a 3 month fallow period. Prior to fallowing, however, chemotherapeutic control (see below) on a bimonthly basis was required to prevent loss of fish from the site due to lice damage.

3.2   Biological Control

One alternative for controlling sea lice infestations currently being investigated is the

use of biological control, specifically cleaner fish which act as anti-lousing predators.

The potential of wrasse as cleaner fish for sea lice control was first demonstrated by

Bjordal (1988) who showed that several species of wrasse, and in particular the

goldsinny (*Ctenolabrus rupestris*) and rock cook (*Ctenolabrus exoletus*), actively

removed lice from infected salmon. Following the results published by Bjordal, several

sea cage trials have been conducted in Norway, Scotland and Ireland and have

confirmed the successful use of wrasse for lice control (Bjordal 1990; 1991; Smith

1990; Costello & Donelly 1991; Treasurer 1991). Stocking densities of wrasse to

salmon (W:S) vary considerably in the literature and range from 1:23 to 1:260 (W:S),

indicating that the effectiveness of wrasse is not always predictable. In most instances

a stocking ratio of 1:50 to 1:100 (W:S) has been reported to be adequate for lice

control. At present the stocking of wrasse in salmon grow-out cages is now routine in

Scotland (Bron & Treasurer 1992) and Norway (in 1991 143,000 wrasse were stocked

in 29 farms in Norway) (Å. Bjordal pers. comm.); however, issues pertaining to

supply, disease risk, optimum stocking densities and the adequate maintenance of

wrasse in cages require further research.

3.3   Vaccines

Another possibility for the control of lice is prevention through vaccination. Although

still in the early stages of research, work based on a cattle tick model (Willadsen 1980)

is currently underway aimed at identifying antigens in salmon to sea lice (Reilly 1990;

Smith 1991). Although immunization of fish against several bacterial pathogens has

experienced wide applications in fish farming (Ellis 1988) the development of a sea

louse vaccine will require many years before it could be used on practical basis.

3.4   Chemotherapy

The chemotherapeutic control of lice in aquaculture has taken on many forms and has ranged from touching lice "with an equal mixture of turpentine and kerosene" (Innes 1926), to suspending bags of garlic or onions in salmon cages (Anon. 1991b). More commonly organochlorine compounds (e.g. dichlorodiphenyl trichloroethane (DDT), benzene hexachloride (BHC), aldrin & dieldrin) were used to treat pond-reared fishes infected with ectoparasites (for reviews see Hoffman & Meyer 1974; Herwig 1979). However, it is now known that many of these compounds can have significant negative effects on the environment, and therefore, are no longer used. With the recent development of mariculture and large-scale production systems capable of holding several million fish, particular consideration must be given to the environmental impact of chemotherapeutants added directly to the sea. Consequently, given the dearth of information on the impact of potential compounds in marine environments, licensing of novel chemotherapeutants is expensive and difficult, making the number of compounds available to salmon farmers extremely limited with some countries prohibiting the use of pesticides in aquaculture (Schlotfeldt 1992).

Before considering a compound for use as a chemotherapeutic agent, efficacy, safety and quality criteria must be satisfied. However, where compounds are added directly or indirectly to the environment, as in the case of feed additives, a lack of serious environmental effects is also required. Several compounds that have been used or evaluated for sea lice control are discussed below with respect to efficacy. A more complete review of sea lice control, including a discussion of environmental implications, is given by Roth, Richards & Sommerville (in press).

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3.4.1 Formaldehyde

Formaldehyde was the first compound reported for use to treat sea lice infestations of farmed salmon in Norway (Johannessen 1974). Treatments were found to be marginally efficacious when the formaldehyde was added to cages, enclosed within a tarpaulin, at a concentration of 400 mg/L. Although juvenile salmon (25 g) could tolerate exposures at this concentration at 2.5°C, mortality was observed at 8.2°C (Johannessen 1974). Given the large quantities required and a low safety margin, the use of formaldehyde to treat lice was not pursued further.

3.4.2 Organophosphates

Organophosphates (OP) were originally used as topical treatments for ectoparasites in freshwater fish culture but more recently represent the class of pesticidal compounds most widely used for the control of sea lice. Their mode of toxicity is to inhibit cholinesterase (ChE) activity in cholinergic nervous systems (Baillie 1985). Malathion, trichlorfon and dichlorvos (DDVP) represent the three OP compounds that have been used either clinically or experimentally to treat sea lice.

3.4.2.1   Malathion

Malathion (S-1,2-bis(ethoxycarbonyl)ethyl O,O-dimethyl phosphorodithioate) was experimentally examined for its toxicity to lice (Høy & Horsberg 1991). Given as a 1 hour bath treatment, a concentration of 5.0 mg/L was required for 100% efficacy in removing lice from infected salmon. However, the treatment was also noted to produce lethargy in treated fish. Thus, due to the compound's low therapeutic margin, no further work was carried out.

3.4.2.2   Trichlorfon

The need for a suitable sea lice chemotherapeutant in Norway led to the development of treatments utilizing trichlorfon (dimethyl 2, 2, 2-trichloro-1-hydroxyethylphosphonate) (Brandal & Egidius 1977; 1979). Initially the compound was experimentally evaluated as an oral treatment, but efficacy was offset by side effects in treated fish, including apparent blindness (Brandal & Egidius 1977). A more effective treatment was developed using trichlorfon as a bath treatment (Brandal & Egidius 1979). The method most commonly employed involved moving the fish into a mobile pen surrounded by a tarpaulin that could be moved around a cage group, allowing the reuse of the treatment solution for several cages (8-10). To reduce stress from crowding, the treatment pen was oxygenated. Initially trichlorfon was used at a concentration of 300 mg/L and exposure periods were varied with temperature. Despite initial success with treatments, unpredictable fish kills (sometimes > 90%) were experienced in individual cases that led the manufacturers to re-evaluate the concentration rate (see below) (Røttereng, Silset, Horsberg & Hektoen 1986; Salte, Syvertsen, Kjønnøy & Fonnum 1987; Horsberg, Høy & Nafstad 1989).

In general, increasing mortalities were encountered during the course of treatment where the same treatment solution was repeatedly used, with the highest mortalities being recorded during the last treatment. Trichlorfon breaks down into the more toxic DDVP, the rate of DDVP production being temperature and pH dependent (Salte *et al.* 1987; Samuelsen 1987). Since it is difficult to estimate the amount of DDVP produced during treatment, lethal levels can occur during prolonged use, and were the cause of the sporadic fish kills. An altered treatment schedule was developed for trichlorfon that accounted for increased efficacy with temperature, with recommended doses

ranging from 300 mg/L at temperatures below 6.0°C to 15 mg/L at temperature between 14.0 and 18.0°C (Horsberg & Høy 1989).

Trichlorfon does not persist in fish tissues, in an active form, following routine treatment for sea lice, with residues falling below 0.001 mg/kg after 12 d (Brandal & Egidius 1979). Mattson, Egidius, Kryvi & Solbakken (1987) reported that, following gastric administration, salmon rapidly metabolized (methyl-^{14}C)trichlorfon via the biliary route and after 14 days most of the radiolabel present in the muscle was identified as water soluble metabolic products. Subsequently, a withdrawal period of 21 days was set by the Norwegian government for trichlorfon when used to treat sea lice (Brandal & Egidius 1979). A slightly longer withdrawal period of 30 days has been set for trichlorfon in Germany (Schlotfeldt 1992).

The total amount of trichlorfon used for aquaculture in Norway was 28.3 metric tons (t) in 1985 which, due to increasing use of DDVP (see below), was reduced to 3.2 t in 1988 (Grave, Engelstad & Søli 1991a). Somewhat higher figures are given by Høy & Horsberg (1991) for 1985 (30.4 t), who report a decrease to 2.4 t in 1990. Under the trade name of NEGUVON* (Bayer)[1], the compound is currently used in eight European countries, although it is only licensed in three (Schlotfeldt 1992). Trichlorfon was previously approved for use in aquaculture in the USA but is no longer available (Meyer 1989). Due to the compound's instability in water and variable degradation to the more toxic DDVP, the use of trichlorfon as an anti sea lice chemotherapeutant has been superseded by commercial preparations based on DDVP.

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[1] Other trade marks include: DIPTEREX*, TUGON* and DYLOX*(U.S.A), all to Bayer AG. and MASOTEN* to Miles Inc.

3.4.2.3 Dichlorvos

Dichlorvos (0,0-dimethyl-2,2-dichlorovinyl phosphate (DDVP)) was introduced to control sea lice during the mid seventies in Scotland (Rae 1979) and is currently the most widely used compound for sea lice control in Europe. DDVP is now currently used in several European countries, but only under full product license in three (U.K., Norway & Iceland) (Schlotfeldt 1992). DDVP was originally available under the trade name NOGOS 50EC* (Ciba-Geigy)[2] which was later replaced by NUVAN 500EC* (Ciba-Geigy), a 50/50 formulation of DDVP and dibutyl phthalate. Subsequently NUVAN* was specifically licensed for use in the U.K. for aquaculture under the tradename AQUAGARD SLT* (Ciba-Geigy) (Buchanan 1992).

It is difficult to estimate the amount of DDVP (or other chemotherapeutants) used to control sea lice and a danger exists where the amount may be extrapolated based on overall production. Thus, figures reported in the literature should be viewed with caution. However, Pike (1989) estimated that 12,000 L of AQUAGARD SLT* (= 6 t DDVP) are used annually in Scotland whereas Grave *et al.* (1991a) estimate that 3.2 t of DDVP were used in Norway in 1988. For 1990, Høy and Horsberg (1991) estimated the Norwegian usage of DDVP at 3.4 t DDVP. Yet in 1990, Norway produced 3 times as much salmon as Scotland in the same year, illustrating that the amount of compound used is more dependent on the overall parasite burden and therefore the frequency of treatment, rather than overall production. Some would argue that the higher cost of DDVP in Norway is a mitigating factor (Høy & Horsberg

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[2] Other trade marks include: Apavap* (KenoGard VT AB), Dedevap* (Bayer) and VAPONA* (Shell Chemical Co.).

1991).

The method used for DDVP treatments is described by Rae (1979) and Wootten *et al.* (1982). The net of the cage to be treated is drawn up to reduce the cage volume and the cage is surrounded by a tarpaulin. The appropriate amount of the formulated product is added to give a final concentration of 1.0 mg/L DDVP. Oxygen diffusers are added to the cage to maintain adequate oxygen levels and to aid the mixing of the compound in the water. After treatment the tarpaulin is removed and the chemical allowed to disperse into the sea. Although both Rae (1979) and Wootten *et al.* (1982) reported the addition of a dye, rhodamine B, to allow monitoring of dispersal of DDVP, rhodamine B was not licensed and is not used. Where water currents are particularly strong, cages are surrounded by a skirt rather than being completely enclosed. In Norway, treatments with DDVP are carried out in the same manner or as described above for trichlorfon (Grave, Engelstad, Søli & Toverud 1991b). However, due to the heavy labour cost involved and, in the case of trichlorfon, the risk of fish kills, the use of tarpaulins and skirts, as described above, has largely replaced the use of the treatment cage (Grave *et al.* 1991a).

To achieve the target concentration of 1.0 mg/L DDVP (= 2.0 mL/L AQUAGARD SLT*), the appropriate volume of AQUAGARD SLT* concentrate, based on the predicted volume of the cage is premixed with sea water and sprayed, poured or pumped into the cage to be treated. However, in practice measurements of DDVP concentrations within cages show stratification and poor mixing with actual concentrations ranging from 0.55 to 3.5 mg/L inside closed tarpaulin type treatments (Wells, Robson & Finlayson 1990), stressing the importance of adequate therapeutic

margins for sea lice chemotherapeutants used in this manner.

DDVP was found to be 100% efficacious in removing adult and pre-adult stages of lice at a concentration of 1.0 mg/L (DDVP) for 1 hour (Rae, 1979). However, as with trichlorfon, DDVP treatment efficacy is temperature dependent (Horsberg, Høy, Djupvik, Hektoen, Hogstad & Ringstad 1987). At higher temperatures (16-17°C), Messager and Esnault (1992) found 100% efficacy using DDVP at 0.5 mg/L for 30 minutes. Due to the effect of temperature on the efficacy of sea lice treatments with DDVP, recommended dose rates in Norway are scaled from 0.5 to 2.0 mg/L (DDVP) depending on ambient sea temperatures at the time of treatment (Horsberg *et al.* 1987). Interestingly, only one dose rate (1.0 mg/L (DDVP)) is permitted by the U.K. licence (Ciba-Geigy 1990).

Work in Scotland has demonstrated that some populations of *Lepeophtheirus salmonis* are resistant to DDVP (Jones, Sommerville & Wootten 1992). These findings are not unexpected, given the dependence on the use of a single compound for sea lice control. Resistance to repeatedly used insecticides in agricultural practice is common, occurring within 2 to 10 years of use (Brown 1986). The obvious implications of resistance are reduced efficacy, resulting in increased dose rates/exposure times with the inevitable result of reduced therapeutic margins. As the therapeutic margin for DDVP is relatively low (see below), such increases in use may make the use of the compound impractical. Possibly of more consequence is the potential for cross resistance to other organophosphorus compounds (Wirth, Georghiou, Pasteur & Luna 1987; Kuwahara 1986) or other classes of pesticides (Fabacher & Chambers 1972; Hamilton, Attia & Hughes 1981), complicating further the slow process of developing replacement

compounds.

Interestingly, DDVP, as with trichlorfon, is ineffective against the larval or chalimus stages (Wootten *et al.* 1982). To optimize treatments, a routine sampling scheme was advised to allow farmers to monitor lice populations and treat fish when the lice population comprised predominantly of post chalimus stages to minimize reinfection from larval stages attached to the fish (Rae 1979).

There are few published reports of DDVP residue studies in salmon following treatment for sea lice. At 4°C, following exposures at 1.0 mg/L (DDVP) (1-h), residues of up to 0.017 and 0.054 μg/g could be detected in muscle and liver tissue, respectively, 3 days after treatment; at 12°C, residues in muscle tissue could only be detected directly after treatment (<0.01 - 0.11 μg/g) and could only be detected at trace levels (<0.01 μg/g) in liver tissues up to 6 days from treatment (Høy & Horsberg 1990). Longer retention times of DDVP in the liver of fish treated at 12°C over those treated at 4°C were believed to be due to individual variation and possibly related to fatty livers in certain individuals resulting in slower metabolic rates and subsequent accumulation. In a similar study, Mattson *et al.* (1987) found that (methyl-^{14}C)-trichlorfon was rapidly eliminated from salmon following intragastric administration with highest levels of radiolabelled material being found in the liver with rapid elimination via the bile. Their studies showed that, after 24 hours, 75% of the administered compound was eliminated and that the compound persisted in muscle tissues primarily as water soluble degradation products of trichlorfon and DDVP. Although longer retention times were found for unidentified residues in blue mussels and European oysters exposed to ^{14}C-labelled trichlorfon (Mattson, Egidius &

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Solbakken 1988), it is believed that, as in salmon, DDVP does not persist in an active form in invertebrate tissues (McHenery, Saward & Seaton 1991). In Norway and Scotland the current withdrawal periods for fish treated with DDVP are 14 and 4 days respectively (Horsberg & Høy 1989; Ciba-Geigy 1990).

The toxicity (96-h LC_{50}) for DDVP to various marine fish and salmonids varies from 0.1 to 3.0 mg/L, the most sensitive tested to date being *Clupea harengus* larvae (0.122 mg/L) (Eisler 1970; Johnson & Finley 1980; Verschueren 1983; McHenery *et al.* 1991). Death is related to acetylcholinesterase (AChE) inhibition, most commonly measured as AChE depression. Depression of AChE in fish is related to several factors that include concentration, length of exposure, species and size of fish and oxygen tension (Weiss 1958; Rath & Misra 1979; Verma, Bansal, Gupta, Pal, Tyagi, Bhatnager, Kumar & Dalela 1982; Høy, Horsberg & Wichstrøm 1991). Recovery occurs by regeneration of AChE, which is dependant on the same factors (Weiss 1958; Zinkl, Shea, Nakamoto & Callman 1987). Raverty (1987) found that fish treated repeatedly at 3 and 6 day intervals (1.0 mg/L DDVP) resulted in cumulative AChE depression, suggesting that fish may become more susceptible to toxic AChE depression if they are not given sufficient recovery periods.

When exposed to DDVP for 1 hour (12°C), Atlantic salmon (*Salmo salar*) were found to tolerate concentrations of 4.0 mg/L but not above (1-h LC_{50} was 8.7 mg/L) (Horsberg *et al.* 1987). Variations in water hardness or in pH do not appear to alter the toxicity of DDVP to salmonids (Johnson & Finley 1980). When death of salmon has occurred from organophosphate poisoning, AChE levels were found to be reduced by 80% or more (Salte *et al.* 1987; Horsberg *et al.* 1989).

In general, toxic signs of stress in fish exposed to DDVP include irregular and erratic movements and convulsive seizures which are often accompanied by leaping from the water (Pal & Konar 1985; Raverty 1987). These behaviours are then followed by signs of loss of balance accompanied by a sticky thread-like substance being exuded from the vent. Chronic exposure has been found to cause a dose dependant decrease in respiration rates (Verma & Tonk 1984). In addition, chronic exposure of singhi (*Heteropneustes (Saccobranchus) fossilis*) to DDVP was found to cause dose dependant increases in lipid peroxidation and decreases in phospholipids in regions of the central nervous system (CNS), both of which are believed to be primary indicators of irreversible brain cell damage (Vadhva & Hasan 1986). Lastly, chronic exposure of *H. fossilis* to DDVP has been correlated to reductions in growth rate (Pal & Konar 1985). Incidental reports from salmon farmers in Scotland indicate that, where the incidence of DDVP treatment is high, poor growth rates can occur (Anon. 1992). Whether such effects, as seen in growth rate, are a result of physiological processes or of stress related to treatment is unknown.

3.4.3 Ivermectin

Originally isolated from the actinomycete, *Streptomyces avermitilis*, avermectins represent the most recently developed groups of insecticides currently used as parasiticides in many veterinary and agricultural (crop) applications. In general, the avermectins act by increasing permeability of chloride ions at inhibitory synapses (Turner & Schaeffer 1989). This effect may be due to release of the inhibitory neurotransmitter δ-amino-butyric acid (GABA), interacting with GABA binding sites, possibly enhancing binding, or by interacting with GABA-independent high-affinity binding sites (Fritz, Wang & Gorio 1979; Mellin, Busch & Wang 1983; Turner &

Schaeffer 1989). Thus, the precise mode of toxicity is as yet unresolved.

Although the compound has not been licensed for use in aquaculture, ivermectin (Merck Sharp & Dohme LTD)[3] has seen widespread use as an in-feed additive to control sea lice in Ireland (Siggins 1990; Clover 1991; O'Sullivan 1991) and has reportedly been used in Scotland (Clover 1991). Initially, ivermectin was experimentally evaluated for use in sea lice control in Ireland in the mid 1980s (Palmer, Rodger, Drinan, Dwyer & Smith 1987). Significant reductions in lice numbers were achieved following a single treatment when administered via the feed at a dose rate of 0.2 mg/kg body weight/day (mg/kg bdy wt/d). When the dose was repeated (3, 17 and 39 days between treatments), similar reductions in lice were recorded and the treated fish appeared free from infection for an extended period of time (60 days). Although it was noted that reinfestation with larval lice was occurring, survival of chalimus stages was impaired. However, the treatment regime and dose rates used were found to have a narrow therapeutic margin (see below). Following these initial trials, modified dose regimens ranging from 0.05 to 0.1 mg/kg bdy wt/d administered on a weekly basis (and in some instance biweekly) over several weeks (or months) was found to confer excellent efficacy in lice control at production sites in Ireland (Smith, Moloney, McElligott, Clark & O'Brien in press). Using one of these modified regimens (0.05 mg/kg bdy wt/d 2x week), O'Halloran et al. (1992) on two separate days within a week, achieving a 98-99% reduction of a heavy infestation of *Ergasilus labracis* in Atlantic salmon smolts. In addition, infections with *Lernaea*, an allied parasitic copepod, have also been treated in goldfish by intramuscular injections

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[3] Trade marks include: CARDOMEC*; EQVALAN*; HEARTGARD 30*; IVOMEC*; ZIMECTERIN* & MECTIZAN*.

of ivermectin at a dose rate of 0.0016 mg/kg bdy wt (Hyland & Adams 1987).

Despite a high octanol-water partition coefficient (1615) (= high lipid solubility) (Halley, Jacob & Lu 1989a), a low bioconcentration factor[4] (54-74) suggests that ivermectin is not expected to accumulate in fishes (Halley, Nessel, Lu & Roncalli 1989b). Although there is no available laboratory derived data on ivermectin bioaccumulation in fishes, the structurally similar abamectin was found not to bioaccumulate in bluegill sunfish, *Lepomis macrochirus* (Wislocki, Grosso & Dybas 1989). The results of this study showed that following a 28 day exposure to abamectin, residues reached a steady state after 10 days and were subsequently reduced by 95% after a 14 day depuration period.

Studies on single oral applications of ivermectin also suggest that the compound is actively metabolized by fish. When ^{3}H-ivermectin was administered orally (single dose) to Atlantic salmon, the compound was found to be slowly absorbed by the gut with the highest concentration of radiolabelled material being found in various tissues (blood, muscle, liver & kidneys) after 4 days (29% of administered dose) (Høy, Horsberg & Nafstad 1990). The concentration of radiolabelled material in these tissues was reduced to 19%, of the administered dose, by day 28. The relative amount of radiolabelled material found in various tissues indicated that the compound accumulated in fatty tissues with the highest relative (to muscle) concentrations being found in the liver. High concentrations were also found in the bile, which was believed to be the main route for excretion. The amount of unchanged drug (as

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[4] See Verschueren 1983; Veith, Macek, Petrocelli & Carrol 1980, for discussions on partition coefficients and bioconcentration factors in fish.

compared to TLC standards) excreted (as a % of the sample taken) was 77% after 1 day and 42% after 15 days, thus suggesting that after 28 days, most of the measured radioactivity probably represent metabolized compound.

There is little available information on the toxicity of ivermectin to fish. For rainbow trout, the respective LC_{50} and NOEC (96-h) values are 3.0 and 0.9 μg/L (Halley et al. 1989a). Despite the overall good efficacy achieved at dose rates of 0.2 mg/kg, Palmer et al. (1987) found that the compound had a narrow safety margin with significant mortalities in fish treated (single dose) at 0.4 mg/kg. O'Halloran et al. (1992) found a 0.6% increase in mortality with more than 3 times as many fish displaying signs of lethargy in smolts (35 g) treated at 0.2 mg/kg. When given as an injection at comparable dose rates, ivermectin was also found to be highly toxic to mottled sculpins (Cottus bairdi) as well as eels (Anguilla anguilla) (Heckman 1985; Taraschewski, Renner, & Mehlhorn 1988).

3.4.4 Pyrethrum

Pyrethrum, a naturally occurring pyrethrin, is an extract from chrysanthemum flowers, and has been used as a pesticide in commercial applications since the early nineteenth century (Bowers 1985). The toxicity of pyrethrum is via interference with the closing of sodium channels in nervous membranes (Baillie 1985; WHO 1989a). Transient increases in sodium permeability result in rapid repetitive impulses in sense organs and sensory nerves (Van den Bercken, Ackkermans & Van der Zalm 1973). Sense organs, sensory fibres and nerve endings are most susceptible to these effects accounting for the compound's ability to rapidly immobilize target pests (commonly referred to as a 'knock down' effect). However, there is a poor correlation between knock down and

toxicity so that the mechanisms leading to acute toxicity are still being resolved (Baillie 1985).

Experimental trials with pyrethrum were initiated in Norway in 1989 (Jakobsen & Holm 1990). For these trials, an oil containing 4% Py-Sal 25* (Norsk Pyrethrum A.S.) (= 1.0% pyrethrum) and 4% piperonyl butoxide[5] was dispersed onto the water surface of a salmon cage. As salmon jumped through the layer of oil, it was presumed that the lipophilic nature of the pyrethrum would penetrate the louse cuticle but not the water soluble mucous layer of fish. To maintain the layer of oil in the cage, the cage was surrounded by a canvas collar which was 100 cm deep, half of this width residing in the water column. Although the duration of treatment was not reported, good efficacy was seen with reductions in adult as well as larval lice. Following the results of these preliminary trials, a series of additional trials was conducted in 1990 to further evaluate the product (Anon. 1991a). In total, eleven trials were conducted, evaluating three different treatment techniques: standard (oil on the water surface with varying thickness in the oil layer), dip and bath. In the standard treatments, oil with 1% pyrethrum was added to the surface of several cages containing fish of different size classes and maintained for a period of five days. Reductions in the mean number of lice on a sample of fish from the cage were seen after two to three days. Efficacy was variable and ranged from 0-70%, the best results being obtained with smolts and an oil layer thickness of 96mm. It is not surprising that the variable jumping activity of the treated fish had a direct effect on efficacy. Although the authors reported that jumping activity could be correlated with lice burden, no data were given. It was also noted that poor

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[5] Although referred to as an anti oxidant by the Jakobsen & Holm (1990), piperonyl butoxide is more correctly referred to as a synergist added to pyrethrins to increase toxicity by inhibiting detoxification pathways.

results obtained in many of the trials may have been due to degradation by light. Reduced efficacy was also noted in two trials that were conducted during a storm.

Dip trials were conducted to evaluate the efficacy of various thicknesses of oil and estimate the number of jumps required for delousing. Dipping anaesthetized fish twice in a 1 cm layer (considered equivalent to one jump) gave a 34% reduction in lice, while six dips gave an 88% reduction in lice. A technique was then developed for use in a commercial capacity where fish were put into cages by being passed through an inclined tube containing a layer of oil. The tubes were mounted to the side of the cage at an angle with 1 m above the water surface. When passed through varying layers of oil with 1% pyrethrum (one pass), anaesthetized fish showed a 37% reduction with a 40 cm layer (equal to 10 L of oil). Interestingly, significant reductions in lice were not achieved with non anaesthetized fish (using 4x volume of oil), suggesting that the use of anaesthetic may have obscured the results by adversely affecting the lice prior to treatment or by increasing the immersion time of the fish in the oil while they were immobilised. Using the same technique, Boxaspen & Holm (1991) treated over 3000 fish with treatment efficacy ranging from 82 to 96%. For bath treatments, anaesthetized fish were immersed for 3 seconds in a solution of Py-Sal which resulted in 90% reduction in lice (Anon. 1991a). Whether the compound is active against larval or chalimus lice is unknown. In the preliminary report by Jakobsen & Holm (1990), a 25% reduction in larval lice is reported for one of the trials, but no detailed observations on the effects were given. None of the other reports on the use of the compound gave details of the effects on larval stages.

The relatively high toxicity of pyrethrum, as well as pyrethroids (the synthetic

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analogues), to fish and invertebrates is believed, in part, to be due to a high density of specific binding sites that are much less prevalent in higher vertebrates (WHO 1989a). However, the high toxicity observed with fish, relative to mammals, may also be due to a low capacity to metabolize such compounds and differences in target organ sensitivity (Glickman, Hamid, Rickert & Lech 1981; Glickman & Lech 1982; Glickman, Weitman & Lech 1982). With respect to salmonids, the acute toxicity of pyrethrum to Coho salmon, *Oncorhynchus kisutch*, and steelhead trout, *O. mykiss*, was determined by Mauck, Olson & Marking (1976) and ranged from 22.5 to 39 μg/L (96-h LC_{50}). In addition, Mauck *et al.* (1976) found that for pyrethrum, as well as several pyrethroids, toxicity was indirectly related to temperature and directly related to pH and water hardness. However, the biological activity of several pyrethroids was not significantly altered by pH, suggesting more stability in the synthetic analogues. With respect to temperature, a similar effect was found in the toxicity of pyrethroids to 3rd instar larvae of *Aedes aegypti* with some compounds being 4 times more toxic at 20°C than 30°C (Cutkomp & Subramanyam 1986).

3.4.5 Carbaryl

Carbaryl (1-naphthyl N-methylcarbamate), a carbamate compound marketed under the trade name SEVIN* (Union Carbide Corp.), is a contact insecticide widely used for the control of agricultural pests. Like OPs, carbamates act by inhibiting cholinesterases. Although there is much data on the environmental impact of carbaryl, much of this data relates to its use in forest management schemes. With respect to aquaculture, carbaryl has been used (since the 1960s), on a restricted basis, for the control of burrowing shrimp (*Calianassa californiensis*, *Upogebia pugettensis*) which cause damage to cultured oyster beds on the Pacific Coast of America (Lindsey 1961; Chambers 1970;

Tufts 1990; WDF/WDE 1992). Recently, carbaryl has been evaluated as a potential chemotherapeutant for sea lice control. Høy and Horsberg (1991) reported 100% efficacy, *in vivo*, after 40 minutes at a concentration of 0.5 mg/L when treating lice infected Atlantic salmon.

Carbaryl does not appear to accumulate in fish and shellfish tissues. However, depuration rates appear to vary depending on the species in question, mode of uptake and detection techniques used, making comparison difficult. When administered orally to Atlantic salmon and cod, ^{14}C-carbaryl was found to be rapidly absorbed by the gut and excreted via the bile (Høy & Horsberg, 1991). Johnson and Finley (1980) found that under conditions of chronic exposure (28 days), channel catfish (*Ictalurus punctatus*) eliminated as much as 8 times more residual compound administered orally than topically following a 28-day depuration period. In contrast, Tomkins (1966) found that 97% of the absorbed carbaryl, following a two-hour bath exposure, was eliminated by pumpkinseed sunfish (*Lepomis gibbosus*) after 8 hours. However, Statham, Pebble and Lech (1975) found a high percentage of unchanged compound in the bile of trout topically treated with ^{14}C-carbaryl 24 hours after exposure.

With respect to acute toxicity, carbaryl is well tolerated in fishes with median tolerance limits (TLm) and 96-h LC_{50} values ranging from 0.8 to 13 mg/L for over 40 species of fishes tested (Stewart, Millemann, & Breese 1967; Macek & McAllister 1970; Post & Schroeder 1971; Johnson & Finley 1980; Verschueren 1983). The 1-h LC_{50} for post smolts (3.5 mg/L) is twelve times that required to kill lice (Bruno, Munro & McHenery 1990). When exposed to concentrations of 1.0 mg/L or higher salmon became restless and began to convulse but recover if placed into fresh sea water (Høy

& Horsberg 1991). Although the toxicity of carbaryl to fish is not influenced by salinity (Katz 1961), depending on the species of fish tested, it will vary with temperature, both directly and indirectly (Johnson & Finley 1980).

3.4.6 Diflubenzuron

Diflubenzuron (1-[4-chlorophenyl]-3[2,6-difluorobenzoyl]-urea) (DFB), an insect growth regulator, represents a very different type of pesticide that has been considered for sea lice control. Unlike the neurotoxins discussed above, DFB acts by inhibiting chitin synthesis, thereby interfering with cuticular formation. Marketed as Dimilin* (T.H. Agricultural & Nutrition Co.), DFB is most widely used in crop protection, primarily for the control of the gypsy moth and boll weevil. With respect to sea lice control, the compound was experimentally evaluated by Høy & Horsberg (1991) who found that a dose rate of 75 mg/kg (bdy wt/d), administered orally to lice infected salmon for a period of 14 days, resulted in significant reductions in both adult and larval stages of lice.

DFB is poorly absorbed by the gut of salmon with peak concentrations, representing 3.75% of the administered dose, found in the blood, muscle, liver and kidneys 12 hours from administration, with a proportion of the compound found to accumulate in cartilaginous tissues (Horsberg & Høy 1991). In their experiments with [^{14}C] diflubenzuron, Horsberg & Høy (1991) also found that the compound was rapidly metabolized and excreted via the bile within 6 h after administration, with 37% of the excreted radioactivity representing the parent compound (as measured by TLC). In the bile, varying concentrations of labelled diflubenzuron (and/or metabolites) were present for a period of 10 days after which they began to decrease significantly.

DFB is relatively non toxic to mammals with LD_{50} values in the range of 4,500 to 10,000 mg/kg (Johnson & Finley 1980; Verschueren 1983). The acute 96-h LC_{50} for rainbow trout (*Oncorhynchus mykiss*) is also relatively high at 140 mg/L (Worthing & Walker 1987). However, DFB is extremely toxic to marine crustaceans with effects ranging from direct mortality to indirect behavioral responses. Mortality and gross morphological deformation in several marine invertebrates, specifically *Rhithropanopeus harrisii*, *Menippe mercenaria*, *Callinectes sapidus*, and *Mysidopsis bahia* was found when they were exposed to DFB at concentrations as low as 0.5 μg/L in static exposures (Christiansen, Costlow & Monroe 1978; Costlow 1979; Nimmo, Hamaker, Moore & Wood 1980). Not so surprisingly, adult crustaceans are more tolerant than larval forms.

3.4.7 Hydrogen Peroxide

Most recently, hydrogen peroxide, H_2O_2, has been considered and evaluated for the control in the Faroes, Norway and U.K. At the time of writing, no published data was available; however, unsubstantiated reports from several individuals indicate that the compound is effective for the treatment of sea lice infestations. To date treatments have been carried out in a fashion similar to that described for dichlorvos (Rae 1979) using a 50% w/v H_2O_2 formulation at a concentration of 1.5 g/L (a.i.), for 20 minutes (Thomassen in press). Although the compound shows good efficacy against adult and preadult lice, toxicity to chalimus stages has not been confirmed. H_2O_2 is toxic to salmon with toxicity increasing with temperature. At low temperatures (6°C) the therapeutic ratio is estimated to be 5; however at higher temperatures (14°C) the therapeutic ratio approaches 0 (J. Thomassen pers. comm.). Damage to salmon exposed to H_2O_2 appears to be restricted to the gills (at concentrations above 3.7 g/L

for 30 min., 6°C), with no damage being observed to the cornea or oesophagus (10 g/L, 30 min., 6°C) (Thomassen in press). Extended exposures (60 min., 6°C) of 1.6 g/L also resulted in histopathological changes in the gill. Despite these limitations, such compounds may prove useful where resistance to pesticides such as DDVP has developed.

3.4.8 Garlic & onions

There are several reports on the use of garlic and onions to control sea lice (Munro 1990; Anon. 1991b; Boxaspen & Holm 1991). Trials involving suspending bags of cut onions in net pens have yet to show any degree of efficacy. Similarly, garlic coated salmon pellets do not appear to reduce lice numbers in salmon (Boxaspen & Holm 1991). However, it was noted that fillets from treated fish had acquired a detectable garlic taint. The use of minced garlic has been shown to be efficacious in controlling *Capillaria sp.* nematodes in carp (Pena, Auro & Sumano 1988). When evaluating such compounds it is important to note that efficacy is highly dependent on the extraction process. A hexane extract was 75% efficacious, as compared to an equivalent concentration of an aqueous extract which showed no anthelmintic effect (Pena *et al.* 1988). In addition, garlic was found to be highly toxic to tilapia at only 4 times the dose rate used to treat carp as mentioned above (Pena *et al.* 1988), stressing the importance of therapeutic margins even for 'natural remedies'.

3.5 Discussion

Where problems with sea lice have become widespread, infestations have historically required several years to establish resident populations that, if left unchecked, can debilitate fish farms. With the growth of the farming of salmon, as well as other fish

species such as sea bass/bream, problems with sea lice are likely to dominate the economic viability of such industries. Given the ubiquitous nature of wild sea lice populations and the speed in which large infestations can develop, the use of chemotherapy presently remains an important component of control strategies.

Despite the need for new, more suitable chemotherapeutic agents for sea lice control, DDVP remains the only licensed compound available to farmers. Due to the lack of available compounds, as well as suitable management techniques, farmers have had to rely on one measure of control, which has inevitably resulted in the selection of resistance. Thus it could be said that in some instances, there are no available treatments for sea lice outbreaks.

Unfortunately, the bulk of the information on sea lice lies in the classical parasitological domain of taxonomy. With respect to aquaculture and the problems associated with sea lice, more research into the biology (physiology, ecology, behaviour, epidemiology) is needed. There are few papers dealing with the biology of sea lice as its relates to chemotherapy. By understanding how different types of compounds affect lice, the most suitable and efficient compounds can be developed.

The purpose of the present study was to identify currently used (or in development) agricultural pest control products which might be suitable for sea lice control. To achieve this goal, the project was divided into 3 phases which included pre-screening, screening and studies on the biology of lice as it relates to chemotherapy.

The purpose of the pre-screening phase was to develop a quick screening protocol to

assess the toxicity of chemotherapeutants to sea lice so that subsequent research could concentrate on the most active (toxic to lice) compounds. Following the pre-screening phase, the second phase was designed to assess the efficacy of selected compounds under laboratory and, in the case of one compound, field, conditions and forms the bulk of the work presented. In addition to gaining necessary information relating to efficacy, additional data was sought on therapeutic ratios and, with respect to organophosphates, the sub-lethal effects to fish following exposure. Although the majority of the studies deal with topical application of chemotherapeutants, the suitability of alternative oral dose regimens was also investigated.

The third phase of the project deals with the biology of lice as it related to chemotherapy. In particular, information was sought on the toxicity of selected chemotherapeutants to chalimus stages of sea lice, which appear to be resistant to dichlorvos. To achieve this aim, laboratory reared populations were used to study lethal and sub-lethal effects of representative (of different pesticide classes) compounds following topical and systemic administration to lice infected salmon. In addition to data pertaining to the effects of chemotherapeutic agents to chalimus stages, infection parameters were characterized and the development of a laboratory reared population from the copepodid to adult was studied.

Lastly, an attempt was made to study the kinetics of uptake and metabolism of pesticides in adult lice. The purpose of these studies was to develop a technique for the study of pesticide pharmacology which could be used in subsequent studies and to possibly gain an insight on lice physiology.

4   A Survey of Alternative Chemotherapeutic Agents for the Control of the Salmon Louse, *Lepeophtheirus salmonis*

4.1   Introduction

As discussed in the preceding chapter, several alternative compounds are currently

being considered as anti-louse chemotherapeutants but none have been commercially

licensed or made readily available to salmon farmers. In 1991, total losses due to sea

lice in Scotland were estimated to be as high as 13% (of total production) despite the

availability of DDVP (Mace 1991). Given the recent development of resistance in

some populations of lice (Jones *et al.* 1992), this figure will undoubtedly increase,

fuelling the need for alternative compounds.

When selecting alternative compounds as potential sea lice chemotherapeutants, several

factors must be considered. Safety to the consumer and the environment are of

primary concern. Equally important, requisite metabolic studies, residue analysis and

ecotoxicological studies are expensive and time consuming. Thus before such studies

are initiated, the initial step in the development of agrochemicals is primary screening

or assessing the toxicity of a given compound to the target pest[6]. Once a compound

has been found to be suitably efficacious, secondary screening is engaged to assess the

toxicity to the animal host (to assess the therapeutic ratio) and other non-target species

(ecotoxicology), pharmacokinetics in the host to assess the levels and fate of residues

and to assess the possible toxicological effects of metabolites in various ecological

systems. Given the large assortment of commercial chemotherapeutants currently

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[6]   Primary screening also includes a summary of the compounds physio-chemical
characteristics.

available for agriculture, a study was initiated to develop a rapid screening process to assess candidate compounds for use as anti louse chemotherapeutants. These preliminary studies employed two *in vitro* techniques depending on the nature of the information sought. The first technique was based on the current DDVP routine sea lice treatment method (Rae 1979; Wootten *et al.* 1982) and was aimed at identifying compounds which showed a higher acute toxicity to lice than DDVP following 1 hour exposure. The second technique employed 24 and 48-h exposures to compare the relative toxicities of selected compounds to lice from different sources. The aims of these studies were to identify DDVP resistant populations and to assess the extent of resistance to other organophosphates (= side resistance) or to other classes of pesticides (= cross resistance). The relative toxicity of several of the test compounds will be discussed in subsequent chapters. No attempt was made to address issues concerning chemical composition, ecotoxicology or pharmacology which are reviewed by Roth *et al.* (in press).

4.2   Methods

4.2.1   Acute toxicity studies: 1-h exposures

Live sea lice (*Lepeophtheirus salmonis*) were collected from various salmon farms on the west coast of Scotland. Lice were collected from Atlantic salmon (*Salmo salar*) killed with a blow to the cranium during routine harvests. Lice were removed from fish with forceps and placed into polythene collecting bags pre-filled with fresh sea water (FSW). The bags were placed into an insulated carrier box and transported back to the laboratory.

Toxicity trials were carried out either 24 or 48 hours from the time of collection.

During these intervals, lice were maintained at a constant temperature of 11.0 ± 1.0 °C in small aerated polystyrene or glass vessels and were given daily water changes with FSW. In some instances where FSW was unavailable, artificial sea water was prepared using "Instant Ocean" sea water salt mix made up to 32‰. All sea water used to maintain lice for the toxicity trials was maintained in a large polypropylene container fitted with air stones, Eheim filter pump and ultra violet sterilizer and was maintained at 11.0 ± 1.0 °C

All experiments were conducted at a temperature of 11.0 ± 1.0 °C and a salinity of 32‰ unless stated otherwise. Lice were divided into three replicate groups of ten containing equal numbers ranging from 5 to 10 adult/preadult lice (♂ and/or ♀) depending on the numbers of lice available. Polystyrene containers were used, each filled with 50 mL pre-cooled FSW. For all of the trials, stock solutions were prepared by adding an appropriate amount of the test compound to FSW. However, in some instances compounds were added to a small amount of ethyl alcohol (ETOH) to aid solubility in FSW. Stock solutions were then serially diluted to prepare working dilutions of compounds to be tested. Since no analysis was made of the actual concentrations tested, results are given as nominal concentrations. Concentrations used for the trials ranged from 0.0001 to 10.0 mg/L depending on the compound tested. Compound names, types, formulations and suppliers used for the 1-h acute toxicity trials are given in Table 4.1. Individual controls were used for each of the trials, in addition to, where necessary, a solvent control. In each instance the solvent control was maintained at a concentration equal to that representing the highest concentration used in the experimental series and never exceeded 0.1%.

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Once all working dilutions of a given compound had been prepared, water in the containers was changed, with the respective dilutions, and allowed to stand for 1-h to simulate recommended sea lice treatment protocols (Ciba-Geigy 1990). After the 1-h exposure period, the solutions in each of the containers were discarded and the containers rinsed two times with FSW and then refilled with FSW. To prevent the accidental loss of lice during rinsing, water was passed through a nylon screen. The containers were maintained for an additional 24-h recovery period.

The response of the lice to treatment was recorded at the end of the 1-h exposure period and after the 24-h recovery period. Lice were examined in the containers by close visual observation and by gentle stimulation with a fine brush. The response was recorded according to the following scale: alive (A) = ability to swim; moribund (M) = movement and/or signs of twitching but an inability to swim; dead (D) = no movement. For analysis, lice which were either moribund or dead were grouped together and considered responsive to the compound. Trials were considered invalid if, after 24 hours, more than one louse in any of the three control or solvent control replicates was either moribund or dead.

4.2.2 Sensitivity studies: 24- 48-h exposures

The sensitivity trials were set up in a similar manner to the 1-h acute toxicity trials with some additional changes.

Exposure periods were 24- and 48-h. After the first 24 hours, solutions in each of the replicates were renewed using freshly prepared stock. Solvent controls were determined to be unnecessary as only water soluble emulsified concentrates or water

soluble compounds were studied. In one instance (DDVP, Fishnish lice), vessels containing lice were aerated throughout the exposure period. Compounds studied included either organophosphates or pyrethroid compounds, which are given in Tables 4.3 - 4.8. The formulations and suppliers of the compounds tested are given in Table 4.1. In some instances only the class to which the compound could be identified is given as the name is the proprietary secret of the respective manufacturer.

Lice were obtained from several sites located in several lochs throughout western Scotland. The locations of the various lochs visited are given in Figures 4.1, 4.2 and in Tables 4.3 - 4.8 (results). Due to restraints as a result of long travel times, many of the sensitivity trials were conducted 'on site'. In many instances, limitations on equipment available meant that trials had to be carried out at a range of temperatures which are given below. At some of these sites, a small portable incubator was used to maintain a constant temperature. FSW water was collected from the loch at the farm sites. Prior to collecting FSW, the salinity of the water was checked to avoid collecting low salinity water and every attempt was made to obtain water free of particulate debris.

The response of the lice was recorded after 24 and 48 hours using the scale as described above. Similarly, lice which were either moribund or dead were considered responsive to the test compound and grouped together for analysis. Trials were considered invalid if, after 24 hours, more than 1 louse was either moribund or dead in any of the control replicates. LC_{50} values and 95% confidence intervals were generated using a BASIC LC_{50} program by probit analysis. Significance of LC_{50} values between sites was determined by examining the overlap of confidence intervals (Greenberg,

Conners, Jenkins & Franson 1980). Response groups used for the calculation for LC_{50} values included one successive 0% and one successive 100% mortality value as outline by Greenberg et al. (1980).

Table 4.1. Names, classes, formulations and suppliers of compounds screened for toxicity to lice *in vitro* (EC = emulsified concentrate, Tech S/L = technical solid/liquid, WP = wettable powder, MS = methanol suspension, % = % active ingredient).

Compound	Formulation	Supplier
Organophosphates		
Azamethiphos	50% w/w WP	Ciba-Geigy
CH50016	11.6% w/v EC	Pitman Moore (Europe)
CH540030	8.0% w/v EC	Pitman Moore (Europe)
CH650003	25.0% w/v EC	Pitman Moore (Europe)
Chlorfenvinphos	85% w/w Tech L	Peter Hand Animal Health
Dichlorvos	50.0% w/v EC	Ciba-Geigy
Pirimiphos methyl	50.0% w/v EC	I.C.I.
Propetamphos	49.85% w/v EC	Border Research Ltd.
Temephos	4.0% w/v EC	Cyanamid U.K.
Pyrethroids		
Lambda-cyhalothrin	87.8% w/w Tech L	I.C.I.
Lambda-cyhalothrin	2.5% E w/v C	I.C.I.
PHRDL-C	10% w/v EC	Peter Hand Animal Health
PHRDL-D	10% w/v EC	Peter Hand Animal Health
Resmethrin	20% w/v MS	Border Research Ltd.
Amidines		
CH540001	12.5% w/v EC	Pitman Moore (Europe)
Avermectins		
Ivermectin	≥98% w/w Tech S	Smith Kline Beecham
Ivermectin	1.0% w/v EC	MSD Agvet
Milbemycins		
SKB1	≥98% w/w Tech S	Smith Kline Beecham
SKB2	≥98% w/w Tech S	Smith Kline Beecham
SKB3	≥98% w/w Tech S	Smith Kline Beecham
SKB4	≥98% w/w Tech S	Smith Kline Beecham
SKB5	≥98% w/w Tech S	Smith Kline Beecham
SKB7	≥98% w/w Tech S	Smith Kline Beecham
Insect Growth Regulators		
PHRDL-B	10% w/v EC	Peter Hand Animal Health
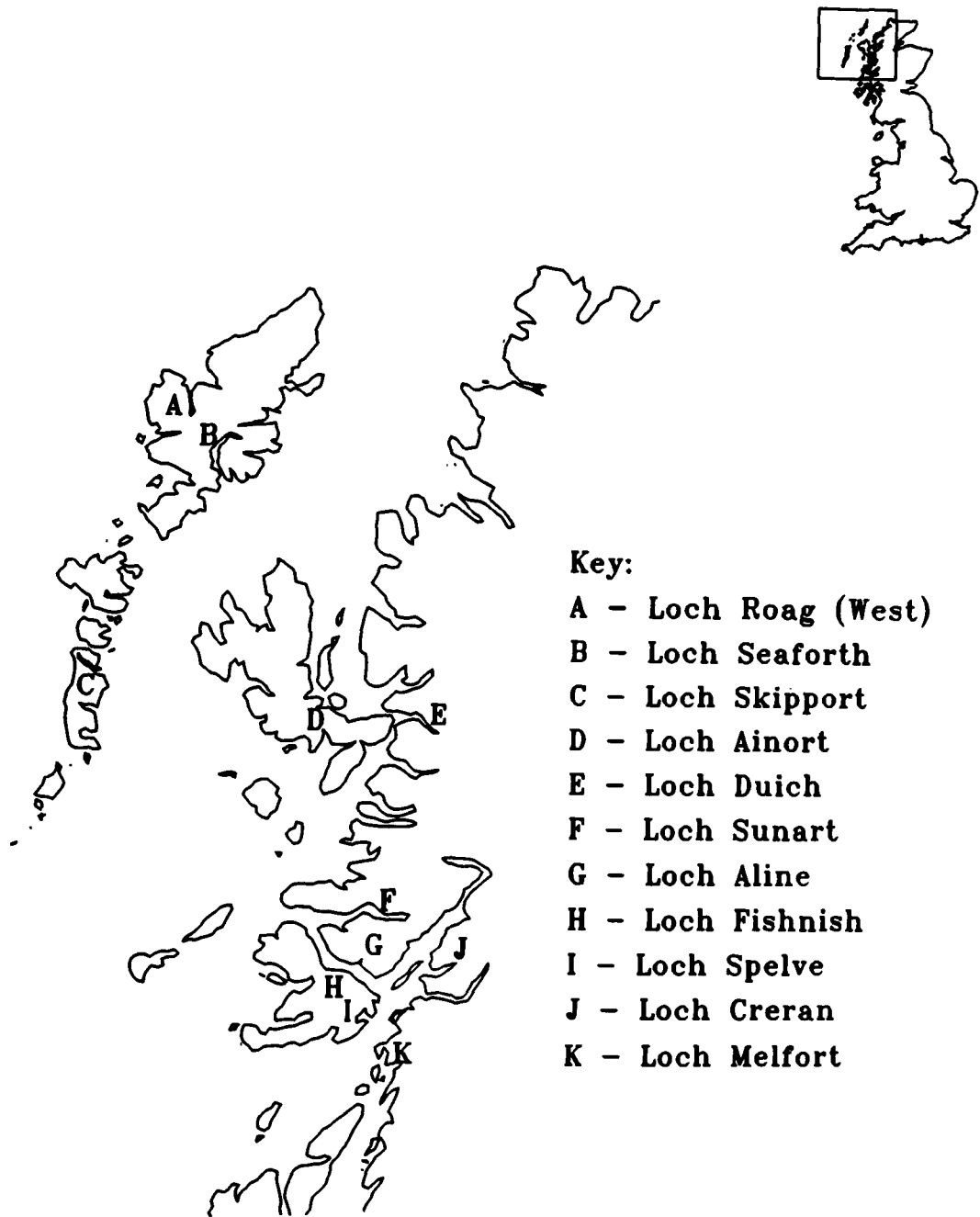
Figure 4.1. Map of the West Coast of Scotland detailing the locations of salmon farms sampled for sea lice.

Key:

A – Loch Roag (West)
B – Loch Seaforth
C – Loch Skipport
D – Loch Ainort
E – Loch Duich
F – Loch Sunart
G – Loch Aline
H – Loch Fishnish
I – Loch Spelve
J – Loch Creran
K – Loch Melfort
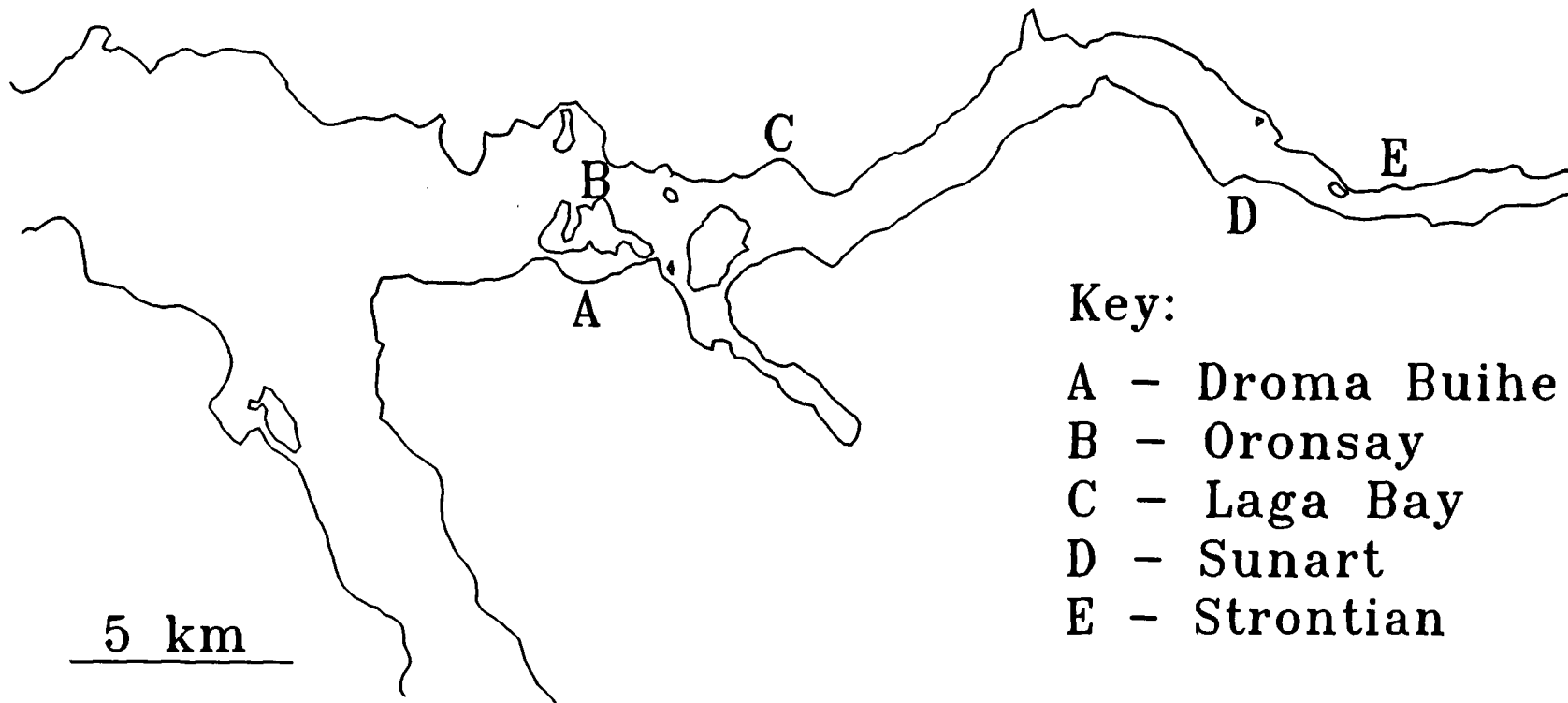
Figure 4.2. Map of Loch Sunart detailing locations of salmon farms sampled for sea lice.

Key:

A – Droma Buihe
B – Oronsay
C – Laga Bay
D – Sunart
E – Strontian

4.3 Results

4.3.1 Acute toxicity trials: 1-h exposures

Generally, it was found that results immediately following exposure were inconclusive with many lice responding to stimuli but dying several hours later; thus results are given for the response of the lice following the 24-h recovery period (= 1+24-h) in Table 4.2. Results in Table 4.2 are given for the dose rate in which 90% or more of the animals tested responded to the compound following the 24 hour recovery period. There was a great deal of variation in the overall toxic range of the 22 compounds tested which spanned 4 orders of magnitude ranging from 0.001 to 10.0 mg/L. Where technical compounds were tested against formulations (lambda-cyhalothrin & ivermectin), no significant differences were found in acute toxicities. No apparent pattern was found with respect to acute toxicity and type of pesticide.

Overall, control survivability was at or near to 100% except in 5 trials (chlorfenvinphos, PHRDL-B/C/D & ivermectin EC). Trials with chlorfenvinphos, PHRDL-B/C & D were repeated on several occasions, but survivability of the control animals was generally poor. Warm summer weather in combination with low salinity sea water at the collection sites (due to heavy rainfall) may have affected the overall survivability of the lice. Trials with ivermectin (EC) were repeated on two occasions when control mortality was 27 and 33%. Given the poor survivability of the controls, acute mortalities of 87 and 67 %, at 0.1 mg/L, for the two trials, more than likely overestimate the acute toxicity of the compound.

Table 4.2. Toxic dose rates ($\geq$ 90% toxicity) for compounds tested *in vitro* against sea lice.

Compound	Effective Dose (mg/L)
Lambda-cyhalothrin (Tech)	0.001
Lambda-cyhalothrin (EC)	0.001
SKB1	0.001
Resmethrin	0.005
SKB7	0.005
Azamethiphos	0.010
PHRDL-C	0.010[1]
SKB2	0.010
SKB4	0.010
PHRDL-D	0.100[1]
Propetamphos	0.100
SKB5	0.100
Ivermectin (EC)	> 0.100[2]
Dichlorvos	1.000
Ivermectin (Tech)	1.000
SKB3	1.000
CH50016	2.000
CH540001	2.000
CH540030	2.000
CH650003	2.000
Temephos	3.000
Chlorfenvinphos	10.000[1]
PHRDL-B	10.000[1]
Pirimiphos-methyl	10.000

[1]Response recorded 6 hours from exposure.
[2]77% mortality at 0.1 mg/L, with suboptimal survivability in the controls.

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4.3.2 Sensitivity studies: 24- 48-h exposures

Many difficulties were encountered maintaining lice over extended periods under static assay conditions. In total, 43 trials were conducted with 9 compounds; 4 OPs and 5 pyrethroids being tested on lice from six lochs. Lochs sampled include: Sunart "A" (Laga Bay), "B" (Droma Buihe), Ainort, Spelve, Aline, Fishnish, Roag and Skipport (see Figures 4.1 & 4.2). However, due to logistical difficulties and salmon harvest schedules, not all the sites could be sampled in a systematic way. Of the 43 trials performed, 24- and 48-h LC_{50} values were determined for only 18 and 9 trials respectively. This was primarily due to poor survivability in control groups. However, in several instances (4 trials after 24 hours and 2 trials after 48 hours), despite 100% survivability in controls, 100% mortality in all the test concentrations prevented LC_{50} determinations. Thus, there was an overall success rate of 42 and 21% in determining 24- and 48-h LC_{50} values respectively. Due to the very low success rate of the 48-h exposures, values are only given for the 24-h exposures which are given in Tables 4.3 - 4.8 and Figures 4.3 - 4.5.

Significant differences in sensitivity to DDVP were most evident between the Sunart A site and the remaining sites studied (Table 4.3, Figure 4.3a). These differences were also evident for azamethiphos tested for toxicity to lice from Sunart A and Loch Fishnish (Figure 4.3b). Despite 100% survivability in controls, lice tested from several other sites (Ainort, Sunart A & B, Aline) also showed high sensitivity to azamethiphos (100% mortality in all test concentrations), such that LC_{50} values could not be estimated (Table 4.4).

Differences between the toxicity of resmethrin to lice from several of the sites did not

appear as pronounced as observed with the OPs tested (Table 4.7, Fig. 4.4a).

Lambda-cyhalothrin also showed marginal site specific toxicity to lice from Lochs

Spelve & Aline (Table 4.8, Figure 4.4b). However, given the low concentrations

required to produce the expected responses for accurate LC_{50} determination, the results

obtained should be approached with caution.

Compounds PHRDL-B/C & D were found to have similar toxicities to lice tested from

lochs Sunart-B and Aline. However a second trial two days later with a group of

freshly collected lice, tested under the same conditions, on site, with compound

PHRDL-B/C & D were found to be significantly less sensitive, demonstrating that a

high degree of variation existed in the experimental system.

Table 4.3. 24-h LC_{50} (μg/L) values for sea lice from different lochs exposed to DDVP (Term = trial terminated).

Date	Lice	Temp.(°C)	24-h LC_{50} (95% CI)
3/7/90	Spelve	10	Term
5/7/90	Spelve	14	Term
24/7/90	Roag	16	Term
30/7/90	Sunart-A	14	20.41 (16.69 - 24.96)
1/8/90	Sunart-B	13	3.10 (1.42 - 6.77)
3/8/90	Sunart-B	13	3.15 (1.44 - 6.90)
3/8/90	Sunart-B	13	Term
14/8/90	Aline	10	2.50 (1.94 - 3.22)
15/8/90	Aline	10	0.34 (0.05 - 2.16)
16/8/90	Aline	10	Term
18/8/90	Fishnish[1]	10	20.60 (14.40 - 29.46)
18/8/90	Fishnish	10	4.94 (3.38 - 7.24)
19/8/90	Fishnish	10	7.82 (5.75 - 10.64)
19/8/90	Fishnish	10	2.18 (1.31 - 3.63)
24/8/90	Skipport	8	3.00 (1.88 - 4.76)

[1]aerated

Table 4.4. 24-h LC$_{50}$ (μg/L) values for sea lice from different lochs exposed to azamethiphos (Term = trial terminated).

Date	Loch	Temp.(^{0}C)	24-h LC$_{50}$ (95% CI)
21/2/90	Sunart-A	10	1.25 (0.93 - 1.67)
21/2/90	Ainort	10	« 0.16
24/7/90	Roag	16	Term
30/7/90	Sunart-A	14	« 0.08
3/8/90	Sunart-B	13	« 0.01
14/8/90	Aline	10	Term
16/8/90	Aline	10	« 0.01
20/8/90	Fishnish	10	0.002 (0.0001 - .02)

Table 4.5. 24-h LC$_{50}$ (μg/L) values for sea lice from Loch Sunart (A) and Loch Aline exposed to chlorfenvinphos and pirimiphos methyl (Term = trial terminated).

Date	Compound	Loch	Temp.($^{\circ}$C)	24-h LC$_{50}$ (95% CI)
30/7/90	chlorfenvinphos	Sunart-A	14	27.21 (14.07 - 52.62)
14/8/90	chlorfenvinphos	Aline	10	Term
15/8/90	Pirimiphos-methyl	Aline	10	2.29 (0.93 - 5.67)

Table 4.6. 24-h LC_{50} (μg/L) values for sea lice from Loch Sunart (B) and Loch Aline exposed to PHRDL-B/C & D.

Date	Compound	Loch	Temp.(°C)	24-h LC_{50} (95% CI)
1/8/90	PHRDL-B	Sunart-B	13	« 0.39
3/8/90	PHRDL-B	Sunart-B	13	0.02 (0.01 - .05)
14/8/90	PHRDL-B	Aline	10	0.05 (0.038 - 0.06)
1/8/90	PHRDL-C	Sunart-B	13	0.22 (0.11 - 0.47)
3/8/90	PHRDL-C	Sunart-B	13	0.008 (0.001 - 0.06)
14/8/90	PHRDL-C	Aline	10	0.06 (0.04 - 0.09)
1/8/90	PHRDL-D	Sunart-B	13	« 0.39
3/8/90	PHRDL-D	Sunart-B	13	0.03 (0.02 - 0.05)
14/8/90	PHRDL-D	Aline	10	0.04 (0.02 - 0.06)

Table 4.7. 24-h LC_{50} (μg/L) values for sea from several different lochs exposed to resmethrin.

Date	Loch	Temp.(°C)	24-h LC_{50} (95% CI)
3/7/90	Spelve	13	0.09 (0.7 - 0.12)
24/7/90	Roag	16	Term
30/7/90	Sunart-A	14	0.77 (0.58 - 1.02)
14/8/90	Aline	10	0.0008 (0.00004 - 0.035)
20/8/90	Fishnish	10	0.04 (0.02 - 0.09)

Table 4.8. 24-h LC_{50} (μg/L) values for sea lice from Lochs Spelve and Aline exposed to lambda-cyhalothrin (Term = trial terminated).

Date	Loch	Temp (°C)	24-h LC_{50} (95% CI)
3/7/90	Spelve	13	Term
5/7/90	Spelve	14	0.001 (0.0005 - .003)
15/8/90	Aline	10	0.010 (0.005 - 0.02)
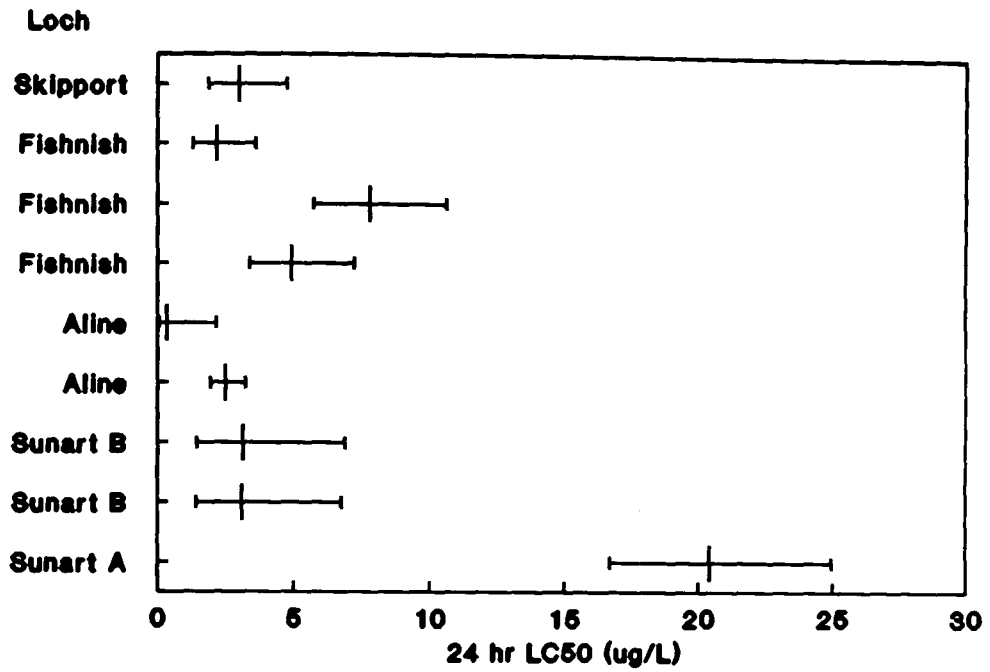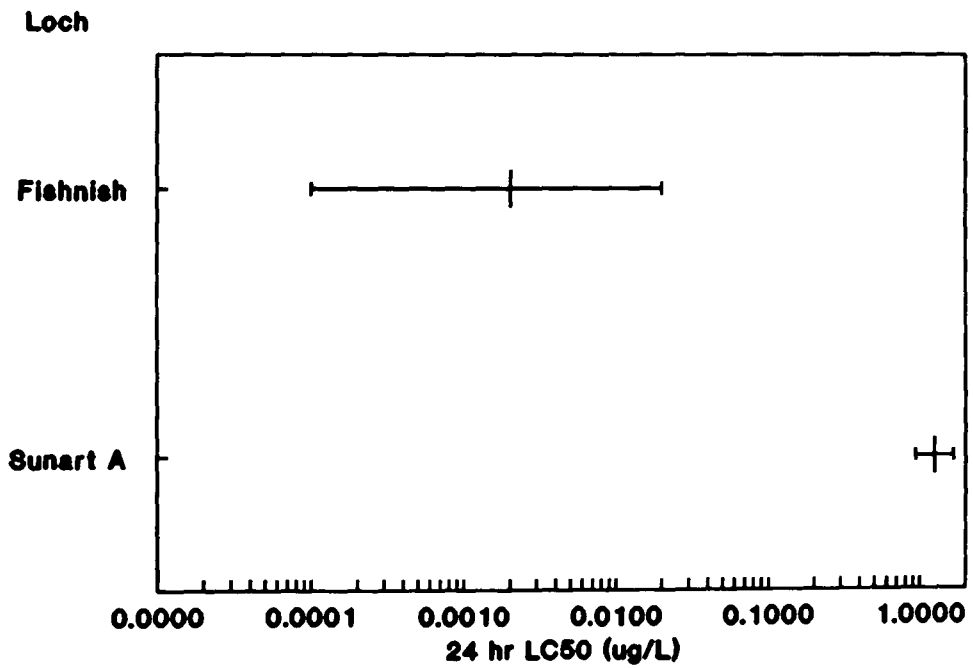
Figure 4.3. 24-h LC$_{50}$ values for DDVP (A) and Azamethiphos (B) following exposure to sea lice from different lochs (bars = 95% CI).

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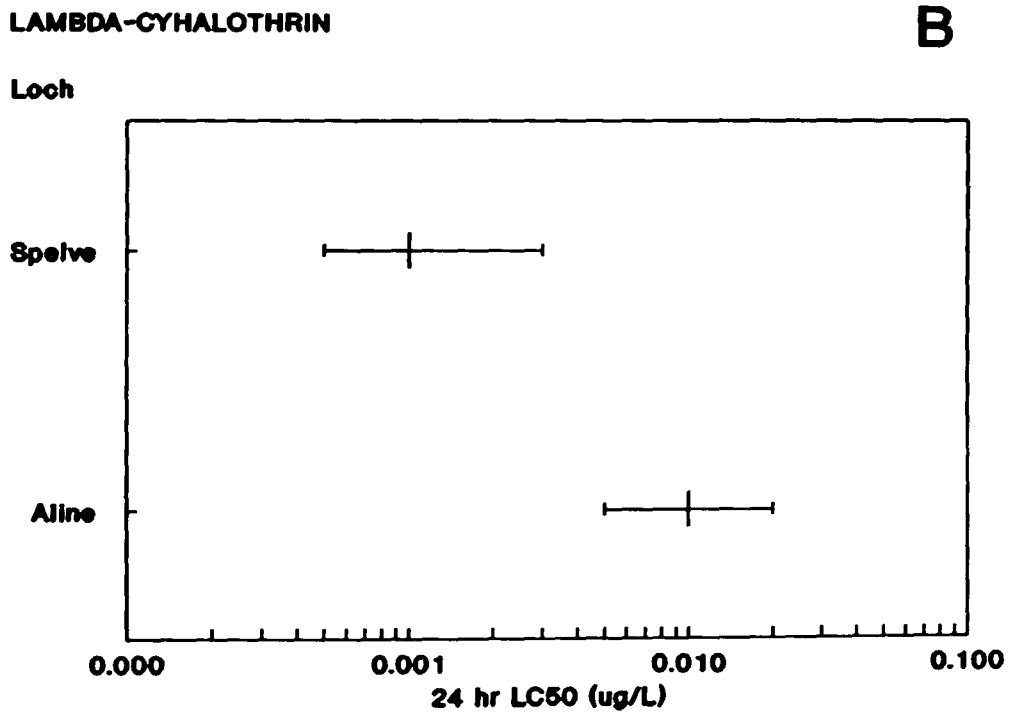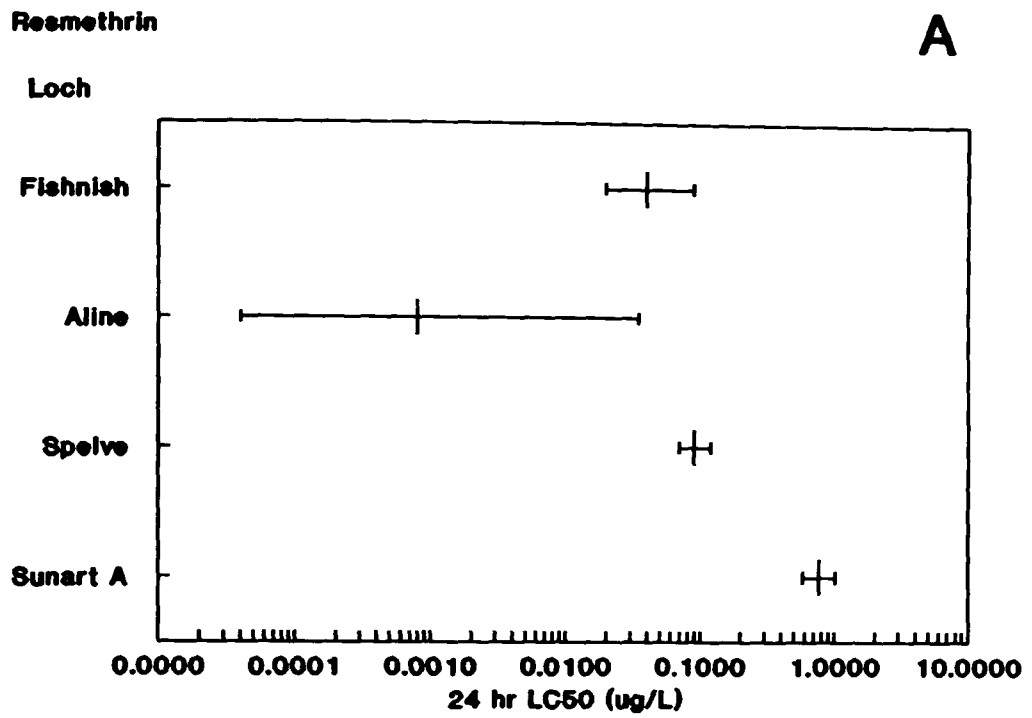
Figure 4.4. 24-h LC_{50} values for resmethrin (A) and lambda-cyhalothrin (B) following exposure to sea lice from different lochs (bars = 95% CI).
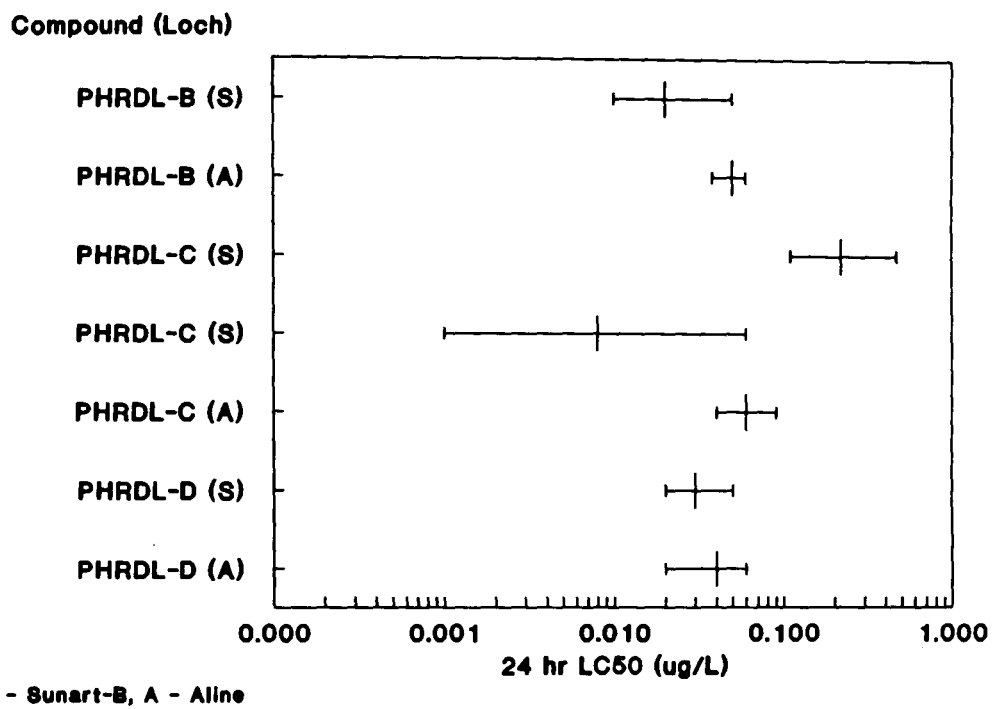
Figure 4.5. 24-h LC_{50} values for PHRDL-B/C & D following exposure to sea lice from different lochs (bars = 95% CI).

4.4   Discussion

It is not surprising that several of the compounds tested, many of which were not OPs, were found to be highly toxic to lice *in vitro*. The dependence on the use of single compounds to control parasitic copepoda in aquaculture will lead, as one might expect, to the development of resistance (Lahav, Shilo & Sarig 1962; Jones *et al.* 1992) and therefore reduced efficacy. Of the compounds found to show high activity towards lice, several different pesticide classes were represented (OPs, pyrethroids, milbemycins). However, in several instances the toxicity of structurally related compounds showed differences of 1 (PHRDL-C & D) or 3 (SKB series) orders of magnitude in toxicity between various formulations, stressing the importance of chemical structure as it relates to target specificity. In some instances, differences in the toxicity of related molecules is related to site specificity (Glickman & Lech 1981) or differential hydrolysis rates of different isomeric configurations (Gray, Conners, Hoellinger & Nguyen-Hoang-Nam 1980; Glickman *et al.* 1981). Thus when screening compounds for activity towards lice, it is essential to screen several structural isomers to identify those which are most active to lice. Furthermore, it would also seem appropriate that, for the future development of alternative chemotherapeutants for sea lice control, compounds with alternative modes of action must be considered so that chemotherapeutic management strategies can be developed (Sawicki 1975; Fergusson-Kolmes, Scott & Dennehy 1991). In particular, the pyrethroid and milbemycin representatives were found to show high activity towards lice and may indicate the potential for both of these pesticide classes for lice control.

Not surprisingly, the IGR, PHRDL-B, was found to have little toxicity towards adult lice. In most instances, IGRs kill juvenile pests by inhibiting chitin synthesis during

moulting or by blocking juvenile development hormones (Christiansen *et al.* 1978; Wilson & Costlow 1986). In adult lice, diphasic growth, characterised by moulting, is replaced by allometric growth which is characterised by increases in the size of the genital complex (Kabata 1979; Anstensrud 1990b). Thus the use of IGRs may be limited to controlling specific (i.e. juvenile) stages of lice. However, in a preliminary trial on the use of the chitin inhibitor diflubenzuron orally in salmon for sea lice control, Høy & Horsberg (1991) reported similar reductions in adult, preadult and chalimus stages.

To date there have been very few studies on the acute toxicity of chemotherapeutants to lice *in vitro* (Bruno *et al.* 1990; Jones *et al.* 1992). In most instances, trial work has concentrated on the effects of chemotherapeutants to lice *in vivo* (see preceding chapter). Although field testing in this manner allows the accurate assessment of efficacy under clinical conditions, it is time consuming and expensive and therefore limits the number of compounds which can be evaluated. Furthermore, no information is obtained on the response of the target pest to compounds in question, nor can various compounds, or experimental parameters (such as population differences), be evaluated.

In many instances, many pesticidal compounds, pyrethroids in particular, have a fast acting or knock down effect (Baillie 1985). In the present study, with all the compounds studied, toxicity to lice appeared to be delayed, commonly referred to as a residual toxicity. Similar findings were reported by Bruno *et al.* (1990) who showed a time dependent response in lice to the carbamate carbaryl. Although the results presented are preliminary, the delayed response may suggest that the kinetics of dispersal of pesticides and subsequent uptake by pest are markedly different in aquatic

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environments. The results also suggest the need for suitable post exposure recovery and observation periods when assessing the toxicity of a given pesticide (Parsons & Surgeoner 1991).

It was also interesting to find that, in the cases of ivermectin and lambda-cyhalothrin, emulsified concentrates were not significantly more toxic than technical compounds. When estimating the toxicity of pyrethrum to the fish louse *Argulus*, Stammer (1959) found that pure compound, when added directly to water, was 50 - 100 % less toxic than equal concentrations of emulsified concentrates. However, whether or not improved solubility or high toxicity of the emulsifying agent was responsible is unknown. In most instances, emulsifiers are added to pesticide formulations to aid penetration in the target pest and thereby increase toxicity (Kumar 1984). One exception may have been the high toxicity of PHRDL-B in the 24- 48-h sensitivity trials, which was found to be relatively non toxic following 1-h exposures, possibly due to the high toxicity of the emulsifying agent rather than the active ingredient (see also below).

The results obtained during the course of the sensitivity trials, although interesting, should be viewed with caution due to the low success rate in maintaining lice for extended periods of time. Several sites sampled were located in remote areas making it impractical to transport lice collected to Stirling. Lice sampled at these remote sights had to be tested "on site" which in most instances proved to be very difficult due to inadequate facilities. Since most of the work was completed during the spring and early summer, it is suspected that low loch salinities coupled with warm air temperatures were the most significant contributing factors to poor survivability of

control animals. However, other factors such as the general condition of the lice may have also contributed to the variation in results. For example, due to the limitations in obtaining lice, it was difficult to standardize lice populations with respect to DDVP exposure on site prior to collection. Thus lice which were collected from a site in which AQUAGARD SLT* had recently been used, may have performed poorly in the bioassays. In future studies variation due to environmental factors and/or management practices may be minimized by establishing laboratory populations for study.

Despite several difficulties, the overall findings of the 24-h sensitivity trials for DDVP are in agreement with values obtained by Jones *et al.* (1992). Although the locations of sites tested were not disclosed, lowest sensitivities were found at sites in Loch Sunart (M. Jones pers. comm.). In their studies, calculated 24-h LC_{50} values ranged from 20 to 36 μg/L for several sites on Loch Sunart. These sites were considered to be infected with less sensitive strains of sea lice when compared to sites which did not have sea lice problems or did not treat with DDVP. Jones *et al.* (1992) reported 24-h LC_{50} values below 7.0 μg/L for sensitive sites. In the present study a 24-h LC_{50} value of 20.4 μg/L was obtained for lice from the Sunart A site (Laga Bay) which was considered to be infected with resistant lice. Similarly, lice obtained from several sites outwith Loch Sunart were found to be relatively sensitive with the few exceptions possibly being the result of experimental error.

Of more concern is the finding that lice which are less sensitive to DDVP are also less sensitive to azamethiphos. Resistance between pesticides from the same chemical class, known as 'side resistance', is common in organophosphates (Kuwahara 1986; Wirth *et al.* 1987), including azamethiphos (Levot & Hughes 1989). Whether resistance

between organophosphates and pyrethroids, known as cross resistance, is present is questionable given the variation in the findings with respect to resmethrin and lambda-cyhalothrin toxicity to lice from sites which show varying levels of resistance to OPs. In many cases, where cross resistance between OPs and pyrethroids has developed (Sawicki 1975; Hamilton *et al.* 1981; Mekuria, Gwinn, Williams & Tidwell 1991), resistance has developed consecutively rather than concurrently. In many instances insect pests which have shown resistance to OPs have been found to be sensitive to pyrethroids (Sawicki 1979; Carter 1989; Levot & Hughes 1989) resulting in the success of the pyrethroids as a dominant class of agrochemicals. Thus the variation in the results may reflect site specific variations in lice populations or inherent variations in experimental technique, rather than the presence of resistance mechanisms. Although efforts were made to used clean filtered sea water, this was not always possible, where trials were conducted on site and variations in organic loading of sea water obtained from the lochs may have reduced the toxicity of the pyrethroids in some instances (Chandler 1990). Thus, before any firm conclusions can be made on the presence of cross resistance in sea lice, further work will be required.

Mechanisms responsible for resistance have been attributed to: delayed penetration (Chaudhry & Price 1990) increases in detoxification enzymes (principally esterases) (Hamilton *et al.* 1981; Ugaki, Abe, Fukami & Shono 1983; El-Khatib & Georghiou 1985) or due to alterations in acetylcholinesterases (Hamilton *et al.* 1981). Resistance to pesticides is also known for vertebrates. Fabacher & Chambers (1972) found tolerant strains of mosquito fish, *Gambusia affinis*, to the pesticide rotenone (a botanical pesticide, obtained from *Derris* roots). The reduced sensitivity was found to be attributed to high levels of mixed function oxygenase (MFO) enzymes, best known

for detoxifying toxins and foreign chemicals (Neff 1985). Interestingly the fish studied by Fabacher & Chambers (1972) had not previously been exposed to rotenone, leading them to conclude that the presence of some other pesticide (they suggested an organochlorine) had induced increased levels of MFO enzymes in the fish thereby conferring cross resistance. The exact nature of resistance in sea lice is, as yet, unresolved. The implications to the salmon farming industry are clear, as introduction of new pesticides would be of little value in developing a control strategy where resistant lice populations occur. Thus, it would be very valuable to understand the nature of the mechanism involved if alterative chemotherapeutants are to be made available and effective.

5 The Efficacy of Alternative Chemotherapeutants for the Treatment of Sea Lice Infections of Atlantic Salmon: Topical Treatments

5.1 Introduction

In the preceding chapter, several novel chemotherapeutants were found which were highly toxic to lice, *in vitro*. However, the suitability of any potential sea lice chemotherapeutant will inevitably depend on the difference between toxicity to the host animal and the target organism, or the therapeutic margin. Therapeutic margins are often discussed as multiples of a given dose rate in which the compound can be used without effect to the host animal. Effects on the host are usually defined in terms of acute toxicity or the absence of cumulative or chronic sublethal effect. The level of therapeutic effect, defined here as efficacy, is more difficult to define and depends on the disease in question (Chou & Chou 1987). Thus, a safety margin of "10" would imply that a chemotherapeutic agent could be used at 10 times the therapeutic dose rate without adverse affect to the host. As previously discussed, dosing rates achieved during routine sea lice treatments can vary by an order of magnitude, thus one would assume that for sea lice control, where bath treatments are employed, a safety margin of 10 would be minimal.

To date, most work reported on the efficacy of various sea lice chemotherapeutants has concentrated on the effect at a single dose, deemed to be the most effective. Although there is a great deal of literature on the use of chemotherapeutics as topical treatments for the control of freshwater parasitic copepods (see Hoffman & Meyer 1974; Herwig 1979 for reviews), there are relatively few reports on the use of chemotherapeutants for the treatment of lice infections with respect to more recently developed intensive

salmonid farming. Topical or "bath" treatments in salmon farming are carried out in a similar fashion as practised by (freshwater) pond farmers, but the size of production sites and the tonnage that can be involved creates logistical problems. Furthermore, current trends in the use of pesticides with respect to disposal in the marine environment severely limits the number of compounds available to farmers. As discussed previously, compounds which have been used, or evaluated, for the control of sea lice infestations include; trichlorfon (Brandal & Egidius 1979; Reyes & Bravo 1983), DDVP (Rae 1979; Horsberg et al. 1987; Messager & Esnault 1991), pyrethrum (Jakobsen & Holm 1990; Anon. 1991a), formaldehyde (Johannessen 1974), malathion, carbaryl and diflubenzuron (Høy & Horsberg 1991). There have also been reports on the use of trichlorfon, although to a much lesser extent, to remove *Caligus* spp. from Japanese Yellowtail, *Seriola quinqueradiata* (Fujita et al. 1968), and Red Drum, *Sciaenops ocellatus* (Landsberg et al. 1991), both reared in sea water. With the exception of the work by Messager and Esnault (1991), who examined the lice response at several concentrations and two treatment regimes (1, 15 and 30 minute exposures) none of the above mentioned studies give details concerning nature of the lice response in relation to dose rate.

Although 1.0 mg/L is the recommended dose rate for DDVP (in the U.K.), it is clear that this may not be applicable to all lice populations. In many instances, much higher dose rates would be required to effectively remove all lice from fish. However, the toxic dose for salmon, when exposed for 60 minutes, is less than 8.7 mg/L (Horsberg et al. 1987). Therefore one can assume that the therapeutic margin of DDVP is, conservatively, approximately 5 times or less. Interestingly, Messager & Esnault (1991), although working at slightly higher temperatures (15-18°C) found that the

therapeutic margin increased with increases in dose rates when used for shorter exposure periods. It is much more difficult to estimate the therapeutic margin for compounds such as trichlorfon which are used in a variety of ways (depending on temperature) and can have profound sub-lethal effects. In the case of pyrethrin, the subjective nature of the dosing, as reported by Jakobsen & Holm (1990), makes it very difficult to estimate therapeutic margins. The remainder of the above mentioned compounds have been only evaluated on an experimental basis, thus information is limiting. For example, when evaluating the potential of carbaryl for use as a sea lice chemotherapeutant, Bruno *et al.* (1990) compared 1-h LC_{50} values determined for lice (*in vitro*) and salmon and estimated that there was a therapeutic ratio of 4, but did not treat infected salmon.

It was the purpose of this phase of the present project to examine in detail the dose response of lice following bath treatment with several, selected (based on *in vitro* toxicity results) chemotherapeutants. In most instances, a range of doses was studied to determine not only effective dose rates for adult and preadult lice, but also effects on salmon, thereby gaining information with respect to therapeutic ratios. Toxicity towards larval lice was not addressed at this time (see Chapter 7). In addition, a field study was conducted with azamethiphos to complement laboratory studies to evaluate the potential of the compound for sea lice control on a commercial basis.

5.2   Methods

5.2.1 Laboratory Trials

In total, 11 compounds were tested for efficacy in killing sea lice on infected salmon when administered as a bath treatment. Compound names, types, suppliers and

formulations tested are given in Table 5.1. In some instances only the class to which the compound could be identified is given as the name is the proprietary secret of the respective manufacturer.

For each trial, Atlantic salmon (*Salmo salar*) were obtained from various sources, including sea water grow-out sites or land based hatcheries, and held for several days in the trial facility for acclimation. Depending on the availability of fish and/or facilities, one of three sites were used. The first site was located at the Sea Life Centre (SLC) near Oban. Tanks used at the SLC were polypropylene circular tanks with a capacity of 540 L and were supplied with flow through sea water pumped from Loch Creran. The second facility used was the Marine Harvest Fish Health Lab located at Loch Ailort. The tanks used at Loch Ailort were square fibreglass tanks with a capacity of approximately 630 L with flow through sea water pumped in from Loch Ailort. The third facility was located at Otterferry Salmon Ltd on Loch Fyne. The tanks at Otterferry were square fibreglass tanks with flow through sea water from Loch Fyne and a capacity of 750 L.

Sea lice (*Lepeophtheirus salmonis* unless stated otherwise) used for the trials were those present on collected fish. However, depending on the current level of infection, or where fish were obtained from a land based facility and lice were not present, live adult and preadult lice were collected and used to infect fish. The locations of the lochs relevant to the origins of the lice stocks used are given in Figures 4.1 & 4.2. Protocols used for collecting lice were those outlined above for *in vitro* testing.

Table 5.1. Names, class, formulation and supplier of compounds tested for *in vivo* efficacy studies (EC = emulsified concentrate; Tech S/L = technical solid/liquid; WP = wettable powder; MS = methanol suspension; % = % active ingredient).

Compound	Formulation	Supplier
Organophosphates		
Dichlorvos	50% w/v EC	Ciba-Geigy
Azamethiphos	50% w/w WP	Ciba-Geigy
CH650003	25.0% w/v EC	Pitman Moore (Europe)
Propetamphos	49.85% w/v EC	Border Research
Milbemycins		
SKB1	>95% w/w Tech S	Smith Kline Beecham
SKB7	>95% w/w Tech S	Smith Kline Beecham
Pyrethroids		
Resmethrin	20% w/v MS	Border Research
Resmethrin	1% w/v EC	Border Research
Lambda-cyhalothrin	87.8% w/w Tech L	I.C.I.
PHRDL-C	10% w/v EC	Peter Hand Animal Health
PHRDL-D	10% w/w EC	Peter Hand Animal Health

Salmon were infected with lice by anaesthetizing them (0.075% benzocaine (ethyl-*p*-aminobenzoate) solution[7]) and placing them in an insulated box filled with FSW, lined with a fine mesh (synthetic) cloth (64 μm). Lice were placed into the liner and allowed to come into contact with fish. Fish were removed from the infection bath when a minimum of 10 lice could be seen attached to them. Once infected, all fish for a particular trial were placed into a single tank and allowed 24 hours to recover. Where fish were not infected, fish were held together overnight to allow them to acclimate. All of the efficacy trials, excluding the time response trial (see below) were conducted as 1 hour bath treatments followed by a 24 hour recovery period to simulate

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[7] by diluting a 10% benzocaine-acetone stock solution

current sea lice treatment practices. Prior to treatment, individual groups of fish were randomly selected and either treated in holding tanks, volume adjusted to a known amount or, in some instances, a large polypropylene tank containing 50 L of FSW. In each case an appropriate amount of stock solution was added to the tank/container, mixed in by gently stirring, and allowed to stand, aerated, for 1 hour. After the exposure period fish were transferred to holding tanks with flow through sea water or, where fish were treated in tanks, the tanks were flushed with fresh sea water whilst being drained to removed the test compound. Stock solutions were prepared as discussed previously. Appropriate controls were used for each compound tested.

After 24 hours fish were sacrificed with a blow to the cranium and enumerated for lice (adults and preadults) and then subsequently weighed. Any fish mortalities incurred as a result of treatment were recorded (see below). In addition to the various efficacy trials, except in the case of DDVP, fish treated with OPs were sampled for brain acetylcholinesterase (AChE) activity. Details pertaining to the compounds tested for AChE activity and the fish sampled are given below. For AChE sampling, fish were sacrificed with a blow to the cranium and the brain excised and placed into Eppendorff tubes and immediately placed on dry ice for transport to the laboratory. Brains were then stored at -20°C until assayed. Brains were stored for a period not exceeding 1 week, thus minimizing any artifactual degradation in enzyme activity (Weiss 1958). Protocols for the brain AChE assay are given below.

In addition to the above, two trials were conducted with azamethiphos, resmethrin and SKB7 to examine the time to response in lice mortality following treatment. Fish used for the trial were obtained from Loch Creran and transferred to the facility at the Sea

Life Centre. Concentrations tested were: azamethiphos (50% w/w WP), 0.3 mg/L; resmethrin (20% w/v MS) 0.1 mg/L; SKB7 (Tech S, solubilized in ETOH) 0.1 mg/L, in addition to a control group. Treatments were 1 hour in length as described above.

For the first trial (Trial 1), fish were divided into groups of 10, treated, and random samples of five fish were then enumerated for lice at each of the sampling points following treatment. For the second trial (Trial 2) fish were divided into groups of 5, treated, and all fish in each group were sampled for lice at each of the sampling points following treatment. To enumerate parasites, fish were netted out of the tanks, anaesthetized in a mild benzocaine solution (0.01%) and inspected by careful visual observation. After the number of lice had been recorded, fish were returned to the appropriate tank. For both trials lice were counted at hourly intervals for the first eight hours following treatment and then at four hour intervals for an additional 16 hours. A final lice count was made 1 week following treatment.

For all trials, efficacy was calculated as a percentage reduction in lice numbers on treated fish relative to lice numbers on control fish.

5.2.2 Field Trials - Azamethiphos

5.2.2.1   Study Sites

Azamethiphos was the only compound tested for efficacy at sea cage grow-out sites under the auspices of an Animal Test Certificate (ATC) granted to Ciba-Geigy Agrochemicals. Sites selected for the field trials included farms located at Lochs Creran, Duich & Seaforth (Figure 4.1). Details pertaining to the location, size, stock and lice sensitivity patterns of each of the sites tested are given in Table 5.2 below. To

reduce the effects of reinfection from adjacent cages, cages not treated as part of the field trials were treated with DDVP at the discretion of the respective site supervisors.

Table 5.2. Site specific details for location, flushing characteristics, number of fish present and lice sensitivity history prior to commencement of azamethiphos field trials for Lochs Creran, Duich and Seaforth. Lice treatments refer to standard AQUAGARD* treatments (= 1.0 mg/L DDVP for 1-h).

	Site		
	Duich	Creran	South Seaforth
Grid Reference	NG895225	NM938422	NB213119
Fresh water/tidal ratio	13.9	12.5	5.5
Runoff width (m^2/day)	713	707	698
Cages	5 groups of 12 x 12m^2 pens + 32 x 5.5 m^2 pens	1 group of 32 x 15 m^2 pens	1 group of 14 x 12 m^2 pens[1]
Fish (year class)	279,246 (1990)	170,000 (1990)	103,615 (1991)
Estimated Biomass (t)	650	415	87
Lice Sensitivity	2 treatments in 1990, 11 treatments in 1991. Lice sensitive to treatments	6 treatments in 1990, 4 treatments in 1991. Lice tolerant to treatment	Fallowed for 5 months prior to stocking, with no treatments prior to field trial

[1]A second group "North Seaforth" (with an additional stock of 102,354 (1991) fish) is located a mile from South Seaforth, but is considered a separate site.

5.2.2.2   Sensitivity Trials

Prior to commencing cage trials with azamethiphos, samples of lice were collected at each of the sites to determine the relative sensitivity patterns of the lice. Lice were also collected from Laga Bay (Loch Sunart) for comparison. Following increasing problems with both sea lice and furunculosis in many of the Loch Sunart sites, the entire loch was fallowed for an intermittent period prior to stocking in 1991. Laga Bay

was fallowed from December 1990 to April 1991. However, fish remained in farms at other sites until February 1991. Thus, although Laga Bay was fallow for 4 months, the entire loch was fallow for approximately 2 months. Following the fallow period at Laga Bay, fish were treated twice in the summer for *Caligus elongatus* infections but *Lepeophtheirus salmonis* had not been recorded at the site in significant numbers prior to the sampling for the sensitivity trial. Fish had not been given any additional treatments prior to the lice sampling for the *in vitro* trials. Thus it was not known what the sensitivity pattern of the *L. salmonis* population was. The same general protocols for *in vitro* lice testing, with some modifications, were used as described above. Groups of 10 lice in 3 replicates were used. Lice were exposed to serial dilutions for 1 hour, without aeration, and then given a 24 hour recovery period, with aeration. LC_{50} values 1-h & 1+24-h were determined by probit analysis.

5.2.2.3 Treatments

Treatments with both azamethiphos and DDVP were carried out using the methods described by Rae (1979). All treatments were one hour long and employed full tarpaulins (also referred to as "bag" treatments) except for one cage, at one of the sites (Loch Creran), where skirts (which only surround the sides of the cage) were used in place of full tarpaulins. All cages were aerated with oxygen during treatments except during skirt treatments where no aeration was used.

Azamethiphos used for the trials was a brown wettable powder (WP) (50% w/w) as supplied by Ciba-Geigy Agrochemicals. AQUAGARD* (50% w/v DDVP) was used for dichlorvos treatments. Azamethiphos was premixed with distilled water at a rate of 200 g/L on shore, and gently shaken for five minutes. The premix solution was then

taken to the cage site and diluted into sea water by adding small aliquots to 25 L of sea water which was subsequently poured into the cage until all of the required premix had been added to the cage. AQUAGARD* was diluted on site as per label instructions.

To standardize the amount of azamethiphos used for a given treatment, all cages of a given size were assumed to have the same *treatment* volume once the cage had been shortened (2m) and enclosed by the tarpaulin. Thus for 12m² cages, treatment volume was estimated at 432.0 m³. Similarly, the estimated volume for 15m² cages was 787.5 m³. Both calculations were based on the assumption that the volume enclosed by the tarpaulin is greater than the hypothetical volume of the cage (ie. 12m x 12m x 2m) due to slack in the tarpaulin below the cage (Wells *et al.* 1990). Thus, although cage depths were reduced to 2m (from 10m) values of 3.0 and 3.5m were used in the volume calculations for the 12 and 15m² cages respectively. Therefore, to achieve the required concentrations of 0.05, 0.1 and 0.2 mg/L azamethiphos, 43, 86 & 172 g were added respectively to 12m² cages and 79, 158 and 316, were added respectively to 15m² cages. For the skirt treatment, at 0.2 mg/L azamethiphos, the volume of the shortened cage was estimated to be 900.0 m³ and 360 g of azamethiphos was used. As with bag treatments, the treatment volume of the skirt treatment was adjusted to account for losses of chemical during the exposure period and a depth value of 4.0m was used for the (skirt) volume calculation. Where cages were treated with DDVP, the volume of AQUAGARD* used was at the discretion of the site manager/veterinary consultant and is given below.

Temperature and salinity were measured at each of the sites during each of the treatments. In addition, where possible, cages were monitored for dissolved oxygen at

15 minute intervals during treatments. For reference, an oxygen reading was also taken in the loch adjacent to the cages. Following treatments, cages were checked for mortalities following treatments by various methods, each of which was site specific and is described below.

5.2.2.3.1   Loch Creran

The site at Loch Creran consists of a single large raft of 40 x 15m² cages (Table 5.2). Fish at the site were 1990 stock. For the present study, 8 pens at the end of the group were given two treatments with azamethiphos, with a sub-sample of the 8 being monitored for efficacy. For the 1st treatment, 3 cages (13, 11 & 9) were treated at 0.2, 0.05 and 0.1 mg/L. An additional 5 cages (15, 7, 5, 3 (skirt) & 1) were treated at 0.2 mg/L. All treatments were of the tarpaulin type except cage 3 where a skirt was used. For the second treatment, all 8 cages were treated at 0.2 mg/L azamethiphos using full tarpaulins except for cage 3 where skirts were used. Mortalities were counted at the Loch Creran site 24 hours following treatment, using "dead baskets". Dead baskets consist of shallow metal trays which lie on the bottom (centre) of the pen and to which access can be gained by a pulley system.

As indicated from the poor efficacy of past AQUAGARD* treatments, the lice were considered to be resistant to DDVP (1 mg/L - 1-h).

5.2.2.3.2   Loch Duich

The site at Loch Duich is composed of 5 rafts, each consisting of 12 cages measuring 12m² in addition to 32 x 5.5m² cages (Table 5.2). Treatments were carried out on the westernmost raft ("group 1") where all of the 12 cages (number 1-12) were treated

three times with azamethiphos (cage 11 excepted, which during the first treatment was treated with DDVP (1.0 mg/L) for comparison). Fish stock in the group 1 raft were from the 1990 year class. Following each treatment a sub-sample of cages was monitored for treatment efficacy. For the first treatment 2 cages (12 & 10) were treated with azamethiphos at 0.05 & 0.1 mg/L (respectively). Cage 11 was treated with DDVP (as stated above). The remaining cages were treated with azamethiphos at 0.2 mg/L. For logistical reasons, fish in cages 9-12 were moved from group 1 to cages located elsewhere on the site following the first treatment. For the second and third treatments all the cages were treated with azamethiphos at 0.2 mg/L. However, it should be noted that, again due to logistical reasons, fish in cages 5 & 6 were treated with DDVP prior to the third scheduled azamethiphos treatment and were not monitored for efficacy. For consistency in the data set, cages 9 & 10, which had been earlier restocked with fish from elsewhere on the site and which had not previously been treated with azamethiphos, were treated and monitored in place of cages 5 & 6. All treatments were 1 hour in length and employed full tarpaulins as described above.

Cages were examined for treatment mortalities 24 hours following treatment. Mortalities were collected via the "dead sock" system which incorporates a receptacle sewn into the bottom of the pen netting, the opening to which can be tied open or closed by a system of guide ropes. During treatments, dead socks were tied shut to prevent fish from being trapped inside. Upon completion of a treatment the sock was tied open when the cage was dropped to its normal depth (9-10m). The sock was then checked the following day for mortalities by lifting one side of the pen net so that site staff could remove any dead fish which had accumulated inside. The protocols practised at the site stipulated that the cage could not lowered until it had been decided,

by the treatment supervisor, that the fish had recovered from treatment - and the treatment hadn't resulted in significant mortalities. Once the cage had been lowered, the sock was left tied open.

Results obtained on the efficacy of DDVP during routine treatment (1.0 mg/L - 1-h) suggested that the lice from this site showed intermediate sensitivity.

5.2.2.3.3   Loch Seaforth

The site at Loch Seaforth consists of two groups of cages. The first group, "North Seaforth", comprises two rafts consisting of 6 x 12m^2 cages and 4 x 15m^2 and was not included in the study. The second group "South Seaforth", located over a mile from North Seaforth and considered a separate site, comprises one raft of 14 cages measuring 12m^2 and was used for the field trials. The cages at South Seaforth were given 2 azamethiphos treatments in total, with a sub-sample of cages being monitored for efficacy. For the first treatment, 3 cages (1, 2 & 3) were treated with azamethiphos at 0.05, 0.1 & 0.2 mg/L. For comparison, cage 4 was treated with DDVP at 1.0 mg/L. The remaining cages in the group were treated with azamethiphos at 0.1 mg/L. For the second treatment 1 cage (8) was treated with azamethiphos at 0.2 mg/L, the remaining cages at 0.1 mg/L. All treatments were 1 hour in length and employed full tarpaulins.

Mortalities at Loch Seaforth were recorded 24 hours following treatments, using the dead sock system as described above.

It was assumed that the lice at Loch Seaforth were fully sensitive to treatment. Fish

stocked at the site had not been treated for sea lice prior to the commencement of the field trials. In addition, the loch was fallowed at the beginning of 1991 for 5 months.

5.2.2.4   Lice Sampling

Cages treated with azamethiphos, and in some instances DDVP, were sampled for lice burdens at four sampling points for each treatment. Sampling points included: 1, prior to treatment (= 0 hours); 2, immediately after treatment (= 1 hour); 3, 24 hours post treatment (= 24 hours) and 4, 168 hours post treatment (= 1 week). Ten fish were sampled at each of the sampling points. Fish were enumerated for adult & pre-adult lice by anaesthetizing fish in a mild benzocaine solution ($\approx$ 0.02%), removing lice with forceps and preserving them in 10% neutral buffered formalin. The number, stage and sex of lice were recorded at the laboratory. However, whilst fish were being sedated, lice were frequently dislodged from the fish and could be seen in the large bin used to anaesthetize fish. To account for these lice, the total number of lice in the bin was counted and recorded. Following enumeration of each group of 10 fish, the sea water in the bin was replaced and any lice remaining removed. Larval lice (chalimus stages) were enumerated, on site, by careful visual examination of sedated fish. No attempt was made to further classify individual larval stages.

Mean numbers of lice per fish in each group (n=10) were calculated by adding the total number of lice counted, including those found in the anaesthetic bath in the case of adult and pre-adult lice, and dividing by 10.

Efficacy of treatment (% reduction), for post treatment samples, was calculated as a percentage reduction from the pre-treatment lice counts using equation 1.

$$100 - ((X_{1,24,168} / X_0) \times 100) = \% \text{ Reduction} \tag{1}$$

where:

$X_{1,24,168}$ = mean number of lice/fish at 1, 24 or 168 hours post treatment

X_0 = mean number of lice/fish in the pre-treatment sample

5.2.3 Acetylcholinesterase Assays

For each of the three sites, one cage was selected to allow monitoring of fish brain acetylcholinesterase (AChE) activity following treatment, except at South Seaforth where an additional cage, treated at a second dose rate, was also sampled. Fish were sampled at four sampling points for each of the treatments which included: 1, a pre treatment sample; 2, a post treatment sample; 3, 24 hours post treatment and 4, 1 week post treatment. However, where second or third treatments took place, an additional group of fish from an adjoining cage not associated with the current treatment was sampled to allow comparisons with the control group. These fish were referred to as the "Out Group". Ten fish were sampled at each of the sampling points. Fish were sampled by sacrificing them with a blow to the cranium and excising the brain. Brains were immediately frozen in liquid nitrogen. Brain samples were stored (where necessary) and then transported in liquid nitrogen to the laboratory where they were stored at -20°C (for a period not exceeding 2 weeks) until assayed. Prior to being assayed for AChE activity, each of the sample groups were subdivided into two groups so that half (n = 5) of the samples could be assayed and the other half (n = 5) stored

(-20°C) for future reference.

For the assay, samples were weighed (wet) and homogenised (50 % w/v) in 100 mM

tris buffer, pH 7.4 (Sigma). The preparations were then centrifuged for ten minutes at

5000 rpm. AChE activity was determined spectrophotometrically by the method of

Ellman, Courtney, Andres and Featherstone (1961) with the following modifications.

The assay mixture contained: 0.1 mL sample (supernatant), 0.85 mL assay mixture (21

mL 100 mM tris, pH 7.4 + 5 mL 5,5'-dithiobis-(2-nitrobenzoic acid (3-carboxy-4-

nitrophenyl disulfide (DTNB)) and 0.05 mL (2.5 mM) acetylthiocholine iodide (Sigma).

The value of the assay was read on a spectrophotometer (Kontron) at 410 nm at 0 and

3 min. Specific enzyme activities were calculated (no. of μmoles of substrate

converted/mL x min) using equation 2 (Pilz 1974).

$$\text{Volume activity} = (\Delta E/\epsilon \times V/v)/\Delta t = [\mu\text{mole/mL} \times \text{min}] \tag{2}$$

where:

ΔE = extinction change (in absorbance)

ϵ = molar estimation coefficient for the product (5-thio-2-nitro-benzoic

   acid) at 410nm (13.6×10^3)

V = assay volume (mL)

v = volume of sample in assay (mL)

Δt = interval between measurements (min)

Using the mean value obtained from the controls as 100% activity, the relative

activities of the treated fish were calculated as a percentage ("Mean % Brain AChE

Activity").

5.3 Results

5.3.1 Laboratory Trials

Data pertaining to the various compounds tested, lice source, experimental conditions (temperature, salinity, weight and number of fish), mean numbers of lice/fish before and after treatments and fish mortalities for each concentration tested are given Tables 5.3 - 5.5. Efficacy results (% reductions) are summarized in Figures 5.1 to 5.10.

Over the course of studies, a dramatic decrease in sensitivity to azamethiphos was found. Initially, when tested against Creran lice, the compound was found to be 100% efficacious at concentrations of 0.01 mg/L (Table 5.3). However, a year and half later, although tested a slightly lower temperatures, optimum concentration had increased to 0.2 - 0.3 mg/L corresponding to a 20 - 30 fold decrease in potency (Figure 5.1a, b & c). Furthermore, at 0.1 mg/L optimum efficacy was not achieved (Creran Lice) until fish had been exposed for 3 hours (Figure 5.2). Overall, lice obtained from loch Aline were found to be less sensitive to azamethiphos than Loch Creran (Figures 5.1c, 5.2), although the difference was marginal. Lice from Loch Melfort were found to be the most sensitive to azamethiphos (Figure 5.1a).

Results from trials with lice from Loch Sunart (Laga Bay) before and after the Sunart fallow period indicated that, although overall lice burden had been reduced for several months, the sensitivity of the lice had not changed appreciably (Figure 5.3).

Data on the acute mortality of azamethiphos to fish is also summarized in Table 5.3. On several occasions fish appeared to tolerate single exposures to concentrations of 0.4 mg/L, in one case 0.5 mg/L, with no or negligible mortalities.

Table 5.3. Experimental parameters, efficacy and fish mortality results for toxicity trials with the organophosphorus compounds azamethiphos, dichlorvos, CH650003 and propetamphos to sea lice *in vivo* (NI = not infected with lice).

Date	T °C	Sal. (‰)	Fish Wt. (g) ± SD	Lice Source	Conc. mg/L (n fish)	Lice Count $X \pm$ SD	Fish Mort.	Comments
Azamethiphos								
1/5/89	10.0	32.0	≈ 1250	Creran	0.0 (5)	42.2 ± 31.6	0	
					0.1 (5)	0.0 ± 0.0	0	
					1.0 (5)	0.0 ± 0.0	0	
25/5/89	11.5	31.0	≈ 1750	NI	0.0 (5)	*	0	Sampled for
					1.0 (5)	*	2	AChE assay
					3.0 (5)	*	5	
12/6/89	11.0	32.0	≈ 1750	Creran	0.0 (5)	17.4 ± 11.5	0	Sampled for
					0.01 (10)	0.0 ± 0.0	0	AChE assay
					0.1 (10)	0.0 ± 0.0	0	
12/9/89	10.0	32.0	≈ 1750	NI	0.0 (10)	*	0	Sampled for
					0.1 (30)	*	0	AChE assay
					0.3 (30)	*	0	- 3 weekly
					0.5 (30)	*	0,4	treatments
					1.0 (10)	*	2	
5/10/90	13.0	27.0	241.0 ± 7.9	Sunart-Laga	0.0 (10)	13.6 ± 4.8	0	
					0.05 (10)	17.2 ± 8.7	0	
					0.1 (10)	10.0 ± 5.1	1	
					0.2 (10)	1.2 ± 1.2	0	
					0.4 (10)	0.0 ± 0.0	1	
					0.8 (10)	0.1 ± 0.3	2	
10/1/91	7.0	30.0	1838.7 ± 117.6	Aline	0.0 (6) 2hr	48.0 ± 10.4	0	
					0.1 (6) 2hr	14.5 ± 4.0	0	
					0.2 (6) 1hr	13.5 ± 7.7	0	
11/1/91	7.0	30.0	800.9 ± 129.3	Creran	0.0 (10)	11.7 ± 4.4	0	
					0.1 (10)	4.1 ± 1.9	0	
					0.2 (9)	2.2 ± 1.7	0	
					0.3 (9)	0.7 ± 1.1	0	
					0.4 (9)	0.0 ± 0.0	0	
11/1/91	7.0	35.0	854.6 ± 188.9	Creran	0.0 (10) 3h	12.4 ± 4.1	0	
					0.1 For:			
					1.5 hr (10)	4.1 ± 1.9	0	
					2.0 hr (10)	3.5 ± 1.5	0	
					2.5 hr (10)	1.6 ± 0.9	0	
					3.0 hr (10)	0.5 ± 0.5	0	

Table 5.3. - Continued

Date	T °C	Sal. (‰)	Fish Wt. (g) ± SD	Lice Source	Conc. mg/L (n fish)	Lice Count $X \pm$ SD	Fish Mort.	Comments
20/5/92	11.5	34.0	94.5	Sunart-Laga	PT (11)	25.1 ± 10.2	0	Post fallow lice collection
					0.0 (10)	29.2 ± 7.3	0	
					0.025 (10)	15.3 ± 5.6	0	
					0.05 (10)	17.3 ± 6.1	0	
					0.1 (10)	11.2 ± 5.1	0	
					0.2 (10)	3.9 ± 1.7	0	
					0.4 (10)	0.4 ± 0.7	0	
28/5/92	12.0	33.0	276.7	Melfort	PT (10)	24.4 ± 14.1	0	
					0.0 (10)	24.4 ± 9.2	0	
					0.0125 (10)	9.6 ± 4.0	0	
					0.025 (10)	3.5 ± 2.5	0	
					0.05 (10)	2.9 ± 2.5	0	
					0.1 (10)	1.1 ± 1.2	0	
					0.2 (10)	0.0 ± 0.0	0	

Dichlorvos

Date	T °C	Sal. (‰)	Fish Wt. (g) ± SD	Lice Source	Conc. mg/L (n fish)	Lice Count $X \pm$ SD	Fish Mort.	Comments
1/10/90	13.0	30.0	220.4 ± 54.7	Sunart-Laga	0.0 (10)	13.1 ± 3.6	0	
					0.5 (10)	8.9 ± 6.1	0	
					1.0 (10)	7.4 ± 0.3	0	
					2.0 (10)	0.3 ± 0.5	0	
					4.0 (10)	0.0 ± 0.0	0	
					8.0 (10)	0.0 ± 0.0	5	
					20.0 (10)	N.I.	10	
16/1/91	7.0	31.0	647.3 ± 143.0	Creran	0.0 (12)	9.4 ± 4.4	0	
					1.0 (6)	7.5 ± 1.9	0	
					2.0 (11)	1.1 ± 0.8	0	
					3.0 (11)	0.0 ± 0.0	0	
					4.0 (9)	0.0 ± 0.0	0	

CH65003

Date	T °C	Sal. (‰)	Fish Wt. (g) ± SD	Lice Source	Conc. mg/L (n fish)	Lice Count $X \pm$ SD	Fish Mort.	Comments
11/8/89	14.0	34.0	≈ 1750	Creran	0.0 (5)	*	0	Sampled for AChE assay. All lice removed in treatment groups.
					1.0 (5)	*	0	
					3.0 (5)	*	0	

Table 5.3. - Continued

Date	T °C	Sal. (‰)	Fish Wt. (g) ± SD	Lice Source	Conc. mg/L (n fish)	Lice Count X ± SD	Fish Mort.	Comments
Propetamphos								
14/6/89	11.0	34.0	≈ 1750	Creran	0.0 (5)	10.2 ± 3.9	0	Sampled for
					0.1 (5)	0.0 ± 0.0	0	AChE assay
					1.0 (5)	0.0 ± 0.0	0	
20/6/89	11.0	34.0	≈ 1750	Creran	0.0 (8)	-	0	Sampled for
					3.0 (5)		0	AChE assay
12/7/89	12.0	33.0	≈ 1750	Creran	0.0 (6)	5.2 ± 4.2	0	Sampled for
					0.1 (6)	0.0 ± 0.0	0	AChE assay
					1.0 (6)	0.0 ± 0.0	0	

At concentrations of 0.8 mg/L or higher, fish were notably stressed and experimental groups suffered from consistent high mortalities in all but one experiment. Fish were found to tolerate 3 weekly repeated exposures at concentrations of 0.3 mg/L but not 0.5 mg/L where 4 of 10 fish died following the second treatment. Interestingly, there were no further mortalities following the third repeated treatment at 0.5 mg/L.

Observations of fish treated with azamethiphos indicated that when chalimus were present, they were unaffected at the concentrations tested. However, in most instances, chalimus infections were highly variable, making it difficult to reach any conclusions on possible toxicity of azamethiphos.
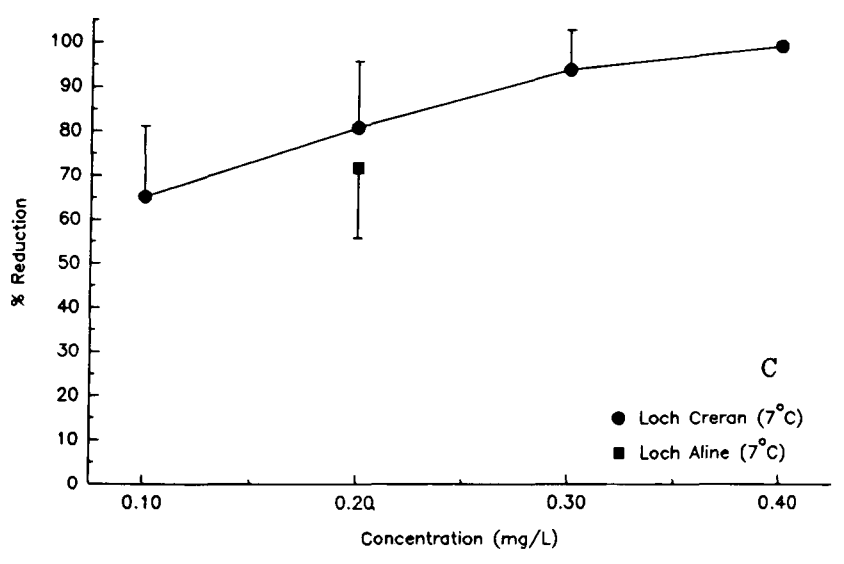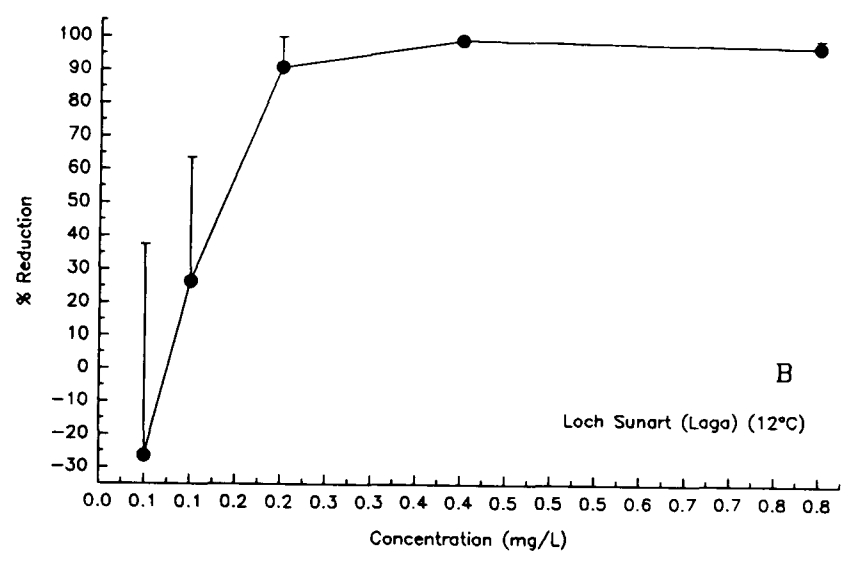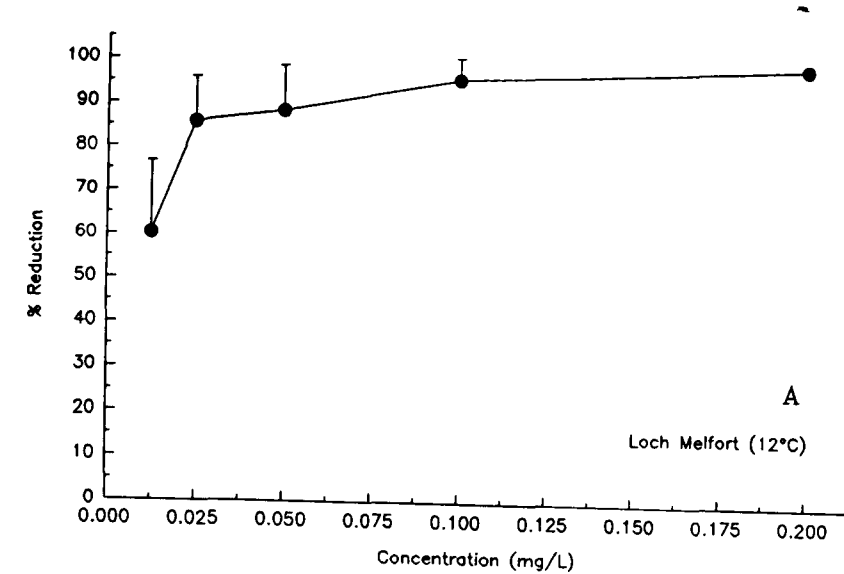
Figure 5.1. Efficacy of azamethiphos against sea lice from different lochs (A = L. Melfort, B = L. Sunart, C = L. Creran & Aline, bars = SD).
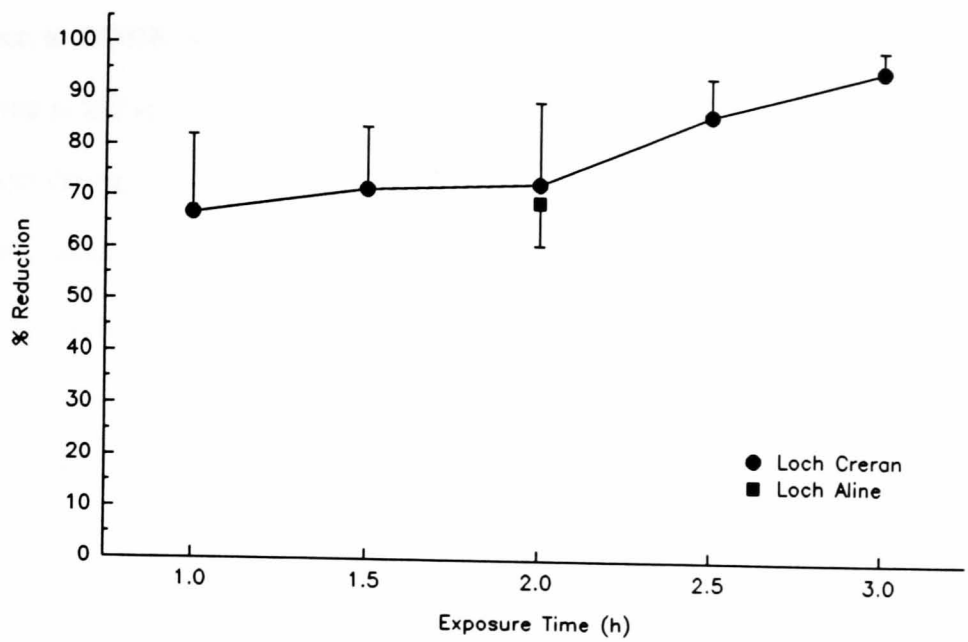
Figure 5.2. Efficacy of azamethiphos (0.1 mg/L) against sea lice (L. Creran & Aline) following extended exposure periods (bars = SD).
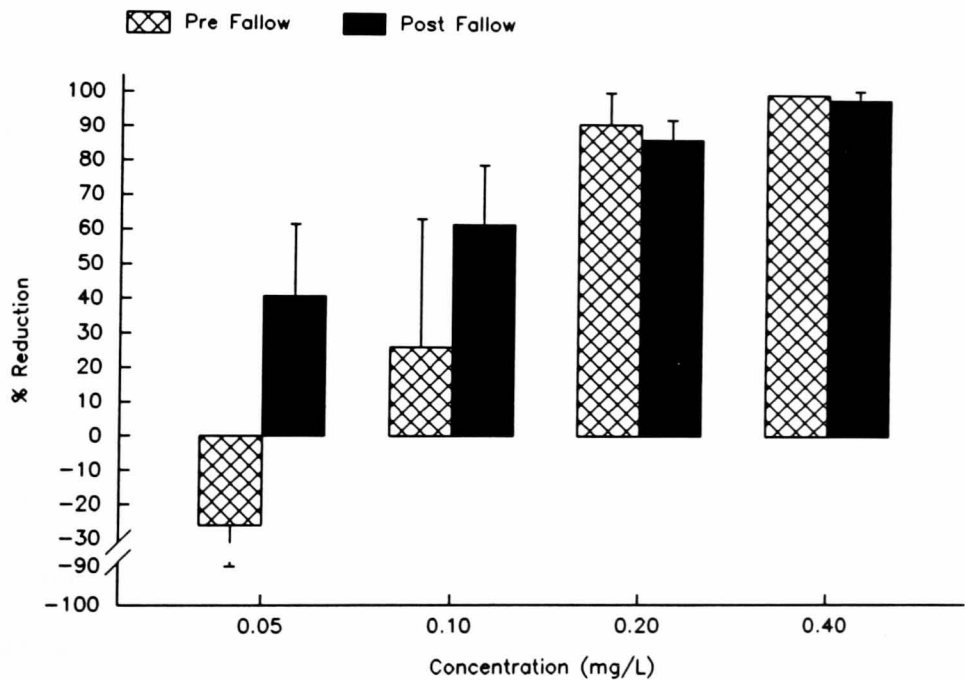

Figure 5.3. Efficacy of azamethiphos against sea lice from Loch Sunart (Laga) before and after the 1991/92 fallow period (bars = SD).

Given that several populations of lice from Loch Sunart have developed various levels of resistance to DDVP, it was not surprising to find that optimum efficacy for DDVP was achieved at 2.0 mg/L rather than one 1.0 mg/L as recommended by the manufacturer (Figure 5.4). Interestingly, temperature appeared to have a marginal effect during treatments with slightly lower efficacy being achieved at 7°C than 13°C. However, any differences may have been masked by different sensitivities of the lice tested, although reports from site managers suggested that, at the time, lice from both Lochs Creran and Sunart (Laga) were displaying pronounce signs of reduced sensitivity. Fish were found to be tolerant of concentrations of 4.0 mg/L with some signs of stress, but not at concentrations tested above (Table 5.3).

Compounds CH650003 and Propetamphos were found to be 100% efficacious at concentrations of 1.0 and 0.1 mg/L respectively. At 0.1 mg/L propetamphos was found to be equally effective following 30 and 60 minute exposures (Table 5.3).

Resmethrin (MS) was found to be highly efficacious at concentrations ranging from 0.01 to 0.1 mg/L. Comparisons between pre-treatment and post-treatment control counts were found not to be significantly different (14/2/90: $t = 0.39$, $P = 0.39$; 8/3/90: $t = 0.34$, $P = 0.74$). In addition, treatments carried out at 0.005 mg/L for 1 hour or 2 hours were not significantly different ($t = 0.58$, $P = 0.57$). In general, initial trials indicated high efficacy at a concentration of 0.01 mg/L, whereas trials carried out 11 months later, with lice from the same loch system, indicated high efficacy at concentrations ranging from 0.05 to 0.1 mg/L depending on experiment conditions (Table 5.4). Results for the dose response trials for lice from Lochs Sunart and Creran are given in Figure 5.5.
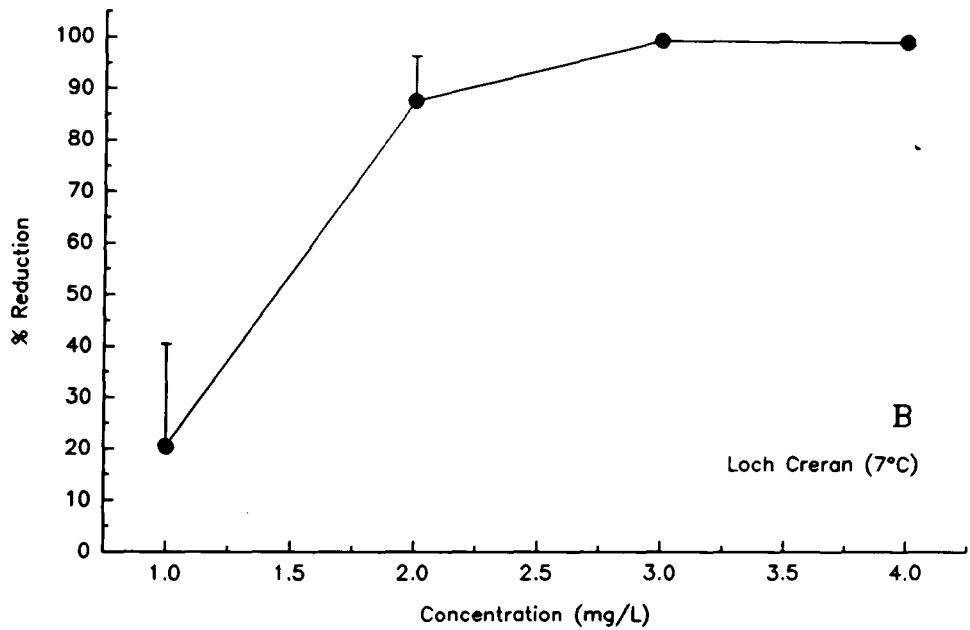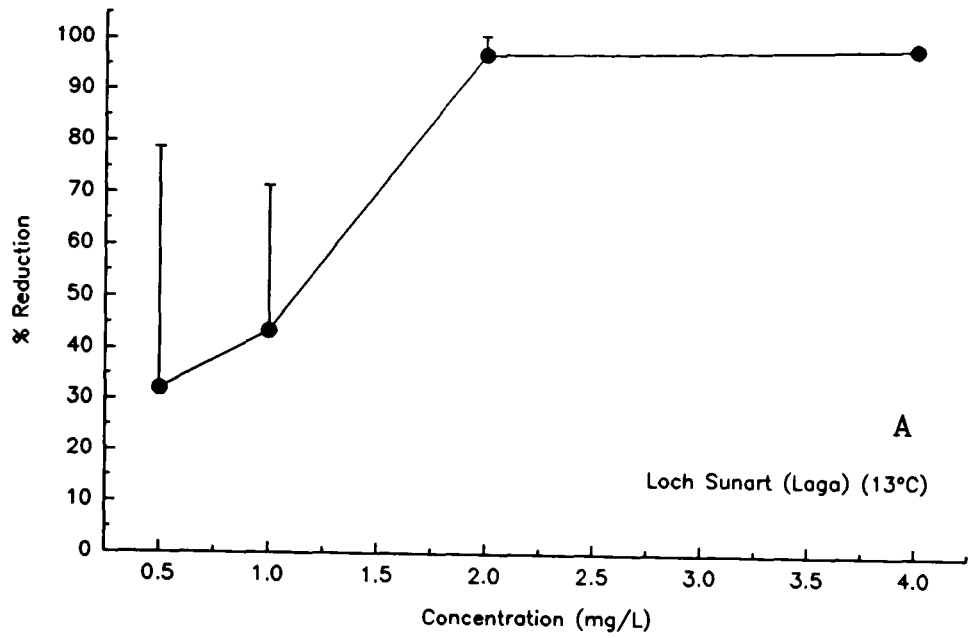
Figure 5.4. Efficacy of DDVP against sea lice from Lochs Sunart (Laga) (A) and Creran (B) (bars = SD).

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Efficacy appeared to be related to temperature. When lice response (Sunart & Creran) at 7 and 15 °C was compared, efficacy was not significantly different at 0.01 mg/L ($t = 0.01$, $P = 1.0$). However, there was a significant difference in efficacy between the two temperatures when tested at 0.05 mg/L ($t = 3.56$, $P < 0.05$) (Table 5.4). There appeared to be little, if any, difference between the efficacy of the two formulations when tested at 0.01 mg/L ($t = 0.07$, $P = 0.05$). However the MS formulation was found to significantly more toxic at 0.05 mg/L ($t = 3.3$, $P < 0.05$) (Figure 5.6). Overall, fish were found to tolerate resmethrin at a dose rate of 1.0 mg/L but were obviously stressed. Although fish could recover from single exposure at 1.0 mg/L, they could not tolerate extended or repeated exposures at this dose rate (Table 5.4).

Lambda-cyhalothrin was only tested once and found to be highly effective at 0.001 mg/L (85% efficacy) and 99 % efficacious at 0.005 mg/L (Figure 5.7). However, the safety margin was found to be low with 100% mortality observed in fish treated at 0.05 mg/L (Table 5.4).

Of all the compounds tested *in vivo*, compounds PHRDL-C & D were found to have the widest therapeutic margins. At 12°C both compounds were highly efficacious at reducing lice at a concentration of 0.001 mg/L (Figure 5.8). Slightly reduced efficacy was achieved at 7°C with Creran lice, however, the slight discrepancy may be artifactual due to differences in the facilities used (Loch Ailort vs Sea Life Centre). The toxicity of both compounds to fish was found to be relatively low with fish tolerating repeated exposures at 0.5 mg/L with negligible mortalities following 3 and 2 exposures with compounds PHRDL-C & D respectively (Table 5.4).

Table 5.4. Experimental parameters, efficacy and fish mortality results for toxicity trials with the pyrethroid compounds resmethrin, lambda-cyhalothrin, PHRDL-C & PHRDL-D to sea lice *in vivo* (NI = not infected with sea lice).

Date	T °C	Sal. (‰)	Fish Wt. (g) ± SD	Lice Source	Conc. mg/L (n fish)	Lice Count $\bar{x}$ ± SD	Fish Mort.	Comments
Resmethrin (MS)								
14/2/90	7.0	32.0	≈ 275	Creran	PT (10)	10.5 ± 4.5	0	silty water
					0.0 (8)	11.4 ± 6.9	0	
					0.1 (10)	1.3 ± 1.0	0	
8/3/90	7.0	32.0	≈ 275	Creran	PT (9)	8.2 ± 2.8	0	3 fish
					0.0 (6)	7.6 ± 3.6	0	jumped in
					0.005 (10)	1.5 ± 1.2	0	0.01
					0.005 (10)	1.2 ± 1.0	0	
					0.01 (3)	0.3 ± 0.5	0 (3)	
11/6/90	14.0	33.5	61.6 ± 9.1	NI	0.0 (20)	*	0/0/1	1 hour
					0.05 (19)	*	0/0/1	exposures
					0.1 (20)	*	0/2/8	3X @ 24
					1.0 (20)	*	0/14	hour intervals
11/6/90	14.0	33.0	65.7 ± 10.4	NI	0.0 (21)	*	0	2 hour
					0.05 (20)	*	0	exposures
					0.1 (20)	*	1	
					1.0 (21)	*	21	
10/9/90	15.0	30.0	360.2 ± 68.8	Sunart-Oronsay	0.0 (10)	11.3 ± 4.1	0	
					0.005 (10)	10.8 ± 5.8	0	
					0.01 (10)	8.4 ± 3.5	0	
					0.025 (10)	5.0 ± 2.3	0	
					0.05 (10)	1.7 ± 1.3	0	
					1.0 (10)	0.0 ± 0.0	0	
14/1/91	7.0	30.5	638.1 ± 133.3	Creran	0 (11)	6.2 ± 2.1	0	
					0.01 (11)	4.6 ± 2.5	0	
					0.05 (10)	0.2 ± 0.4	0	
					0.1 (10)	0.0 ± 0.0	0	
					0.5 (10)	0.0 ± 0.0	0	
Resmethrin (EC)								
5/2/91	7.0	35.0	2123.8 ± 896.5	Aline	0.0 (6)	24.8 ± 13.9	0	
					0.01 (6)	16.0 ± 7.3	0	
					0.05 (6)	6.0 ± 4.5	0	

Table 5.4. -Continued

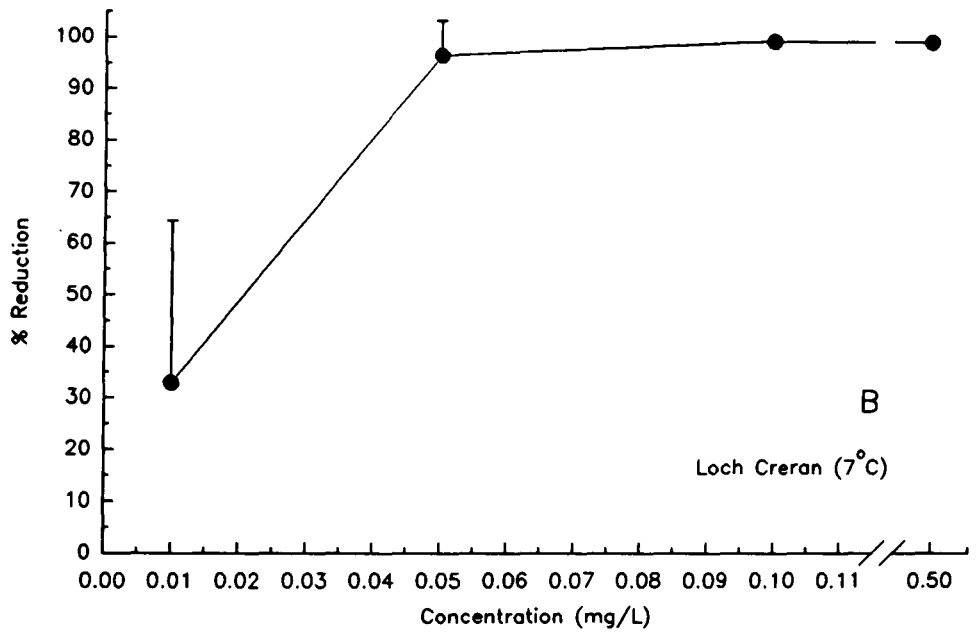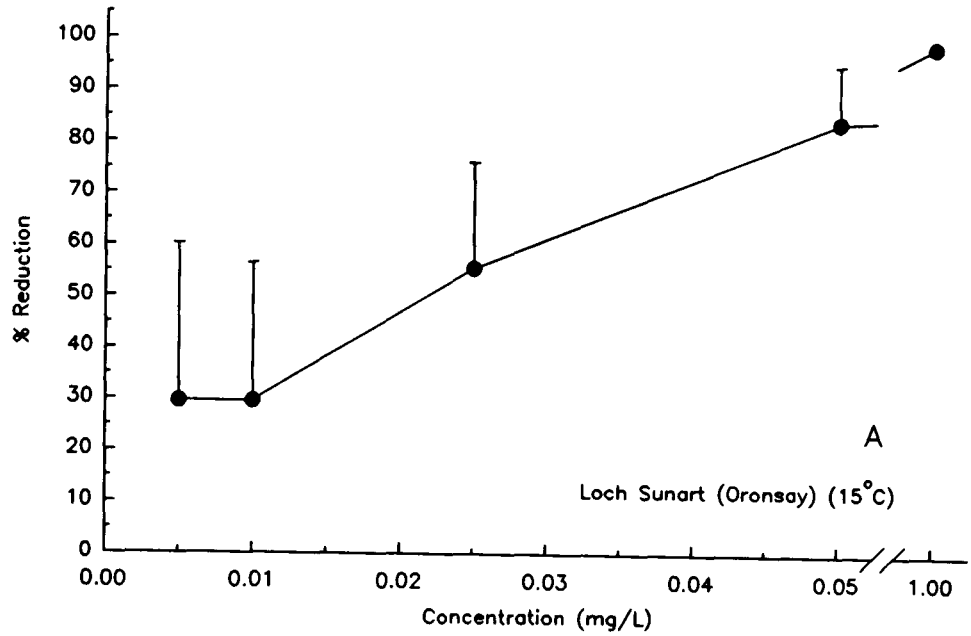| Date	T °C	Sal. (‰)	Fish Wt. (g) ± SD	Lice Source	Conc. mg/L (n fish)	Lice Count X ± SD	Fish Mort.	Comments
Lambda-cyhalothrin								
10/9/90	15.0	30.0	341.0 ± 56.1	Sunart-Oronsay	0.0 (12)	13.0 ± 4.2	0	
					0.0005 (10)	7.6 ± 3.8	0	
					0.001 (10)	2.0 ± 1.5	0	
					0.005 (10)	0.1 ± 0.3	0	
					0.01 (10)	0.0 ± 0.0	0	
					0.05 (10)	0.0 ± 0.0	10	
PHRDL-C								
17/10/90	11.0	17.5	465.9 ± 83.1	Sunart-Strontian	PT (9)	26.8 ± 14.9	0	
					0.0 (8)	14.9 ± 6.9	0	
					0.01 (8)	0.1 ± 0.3	0	
					0.05 (8)	0.0 ± 0.0	0	
					0.1 (8)	0.0 ± 0.0	0	
19/10/90	11.0	24.0	≈ 450	NI	0.0 (8)	*	0/0/0	1 hr exposure 3x @ 24 hour intervals
					0.5 (8)	*	0/0/1	
20/10/90	12.0	24.0	479.0 ± 58.5	Sunart-Laga	PT (8)	26.9 ± 10.6	0	
					0.0 (9)	26.7 ± 8.0	0	
					0.001 (7)	2.1 ± 1.7	0	
					0.005 (7)	0.0 ± 0.0	0	
					0.01 (7)	0.0 ± 0.0	0	
PHRDL-D								
17/10/90	11.0	17.5	446.3 ± 75.6	Sunart-Strontian	PT (9)	26.8 ± 14.9	0	
					0.0 (8)	14.9 ± 6.6	0	
					0.01 (7)	0.0 ± 0.0	0	
					0.05 (8)	0.0 ± 0.0	0	
					0.1 (8)	0.0 ± 0.0	0	
19/10/90	11.0	24.0	≈ 450	NI	0.0 (8)	*	0/0/0	1 hr exposure 3x @ 24 hour intervals
					0.5 (8)	*	0/1/1	
20/10/90	12.0	23.0	495.5 ± 70.7	Sunart-Laga	PT (9)	26.9 ± 10.6	0	
					0.0 (9)	26.7 ± 8.0	0	
					0.001 (7)	1.1 ± 0.8	0	
					0.005 (7)	0.0 ± 0.0	0	
					0.01 (7)	0.0 ± 0.0	0	
14/1/91	7.0	31.0	659.7 ± 126.2	Creran	0.0 (11)	6.2 ± 2.2	0	
					0.001 (11)	1.0 ± 1.0	0	
					0.002 (11)	0.5 ± 0.9	0	
					0.003 (11)	0.5 ± 0.5	0	
					0.004 (11)	0.2 ± 0.4	0	
					0.005 (11)	0.0 ± 0.0	0	

Figure 5.5. Efficacy of resmethrin (MS) against sea lice from Lochs Sunart (Oronsay) (A) and Creran (B) (bars = SD).
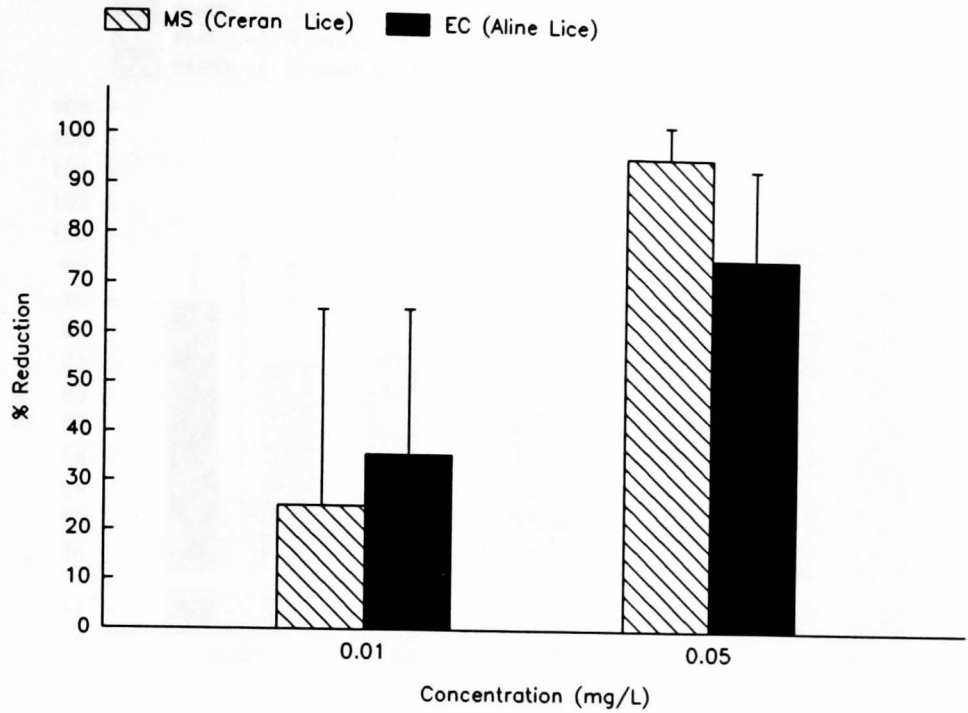
Figure 5.6. Efficacy of two formulations of resmethrin against sea lice (7°C) (MS = methanol suspension, EC = emulsified concentrate, bars = SD).
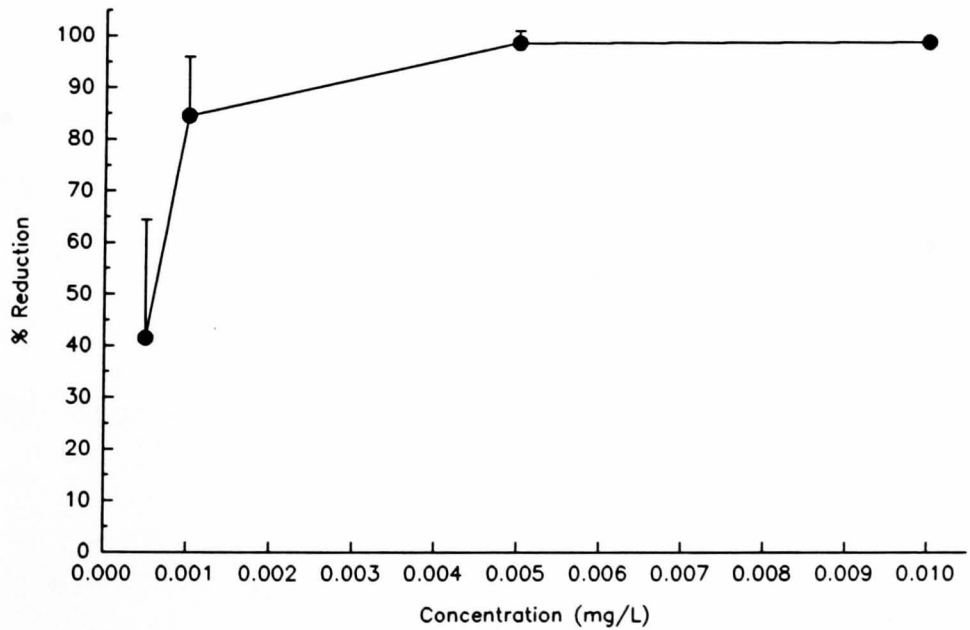

Figure 5.7. Efficacy of lambda-cyhalothrin against sea lice (Sunart, Oronsay; 15°C) (bars = SD).
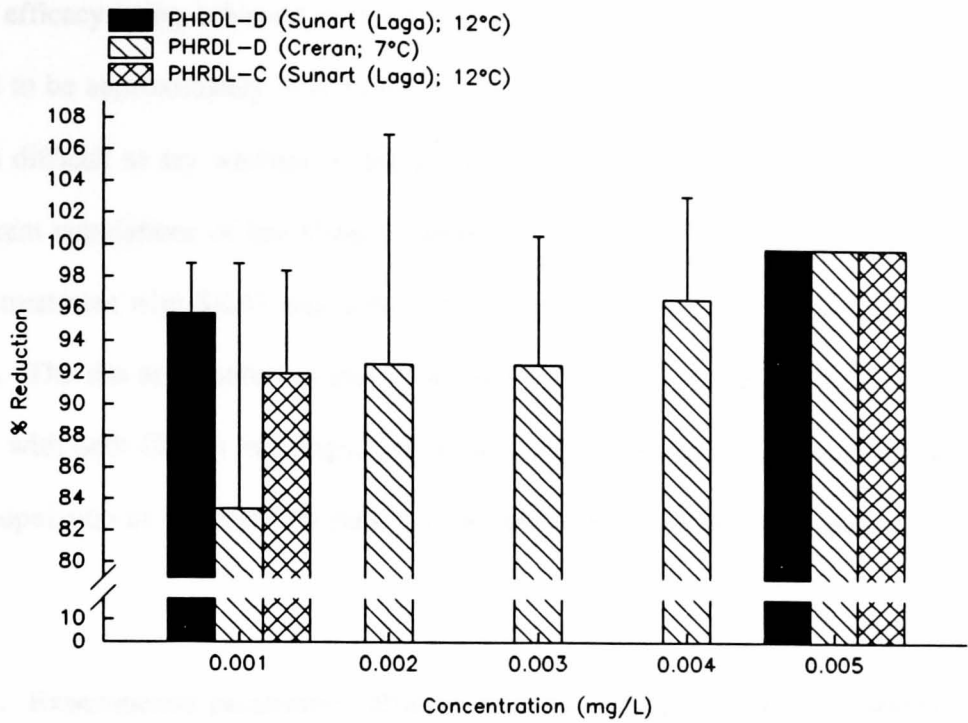
Figure 5.8. Efficacy of PHRDL-C & -D against sea lice from Lochs Sunart (Laga) & Creran (bars = SD).

Of the milbemycins tested, SKB1 was 100% efficacious at 0.01 mg/L (Creran lice)

with 79% efficacy being achieved at 0.001 as well as 0.005 mg/L (Figure 5.9a). SKB7

was found to be approximately 5-10 times less toxic than SKB1 (Figure 5.9b, Table

5.5). It is difficult to say whether or not temperature had an effect. Although tested

with different populations of lice (Sunart (Strontian) vs Creran) at 0.1 mg/L, efficacy

following treatment with SKB7 was 60% and 20% at 14 and 7°C respectively (Figures

5.9b & c). The site at Strontian is located at the head of the loch several miles from

Laga. As with both Creran and Laga, lice at Strontian were believed to represent a

resistant population as indicated by poor success with routine DDVP treatments.

Table 5.5. Experimental parameters, efficacy and fish mortality results for toxicity trials with the milbemycin compounds SKB1 and SKB7 to sea lice *in vivo*.

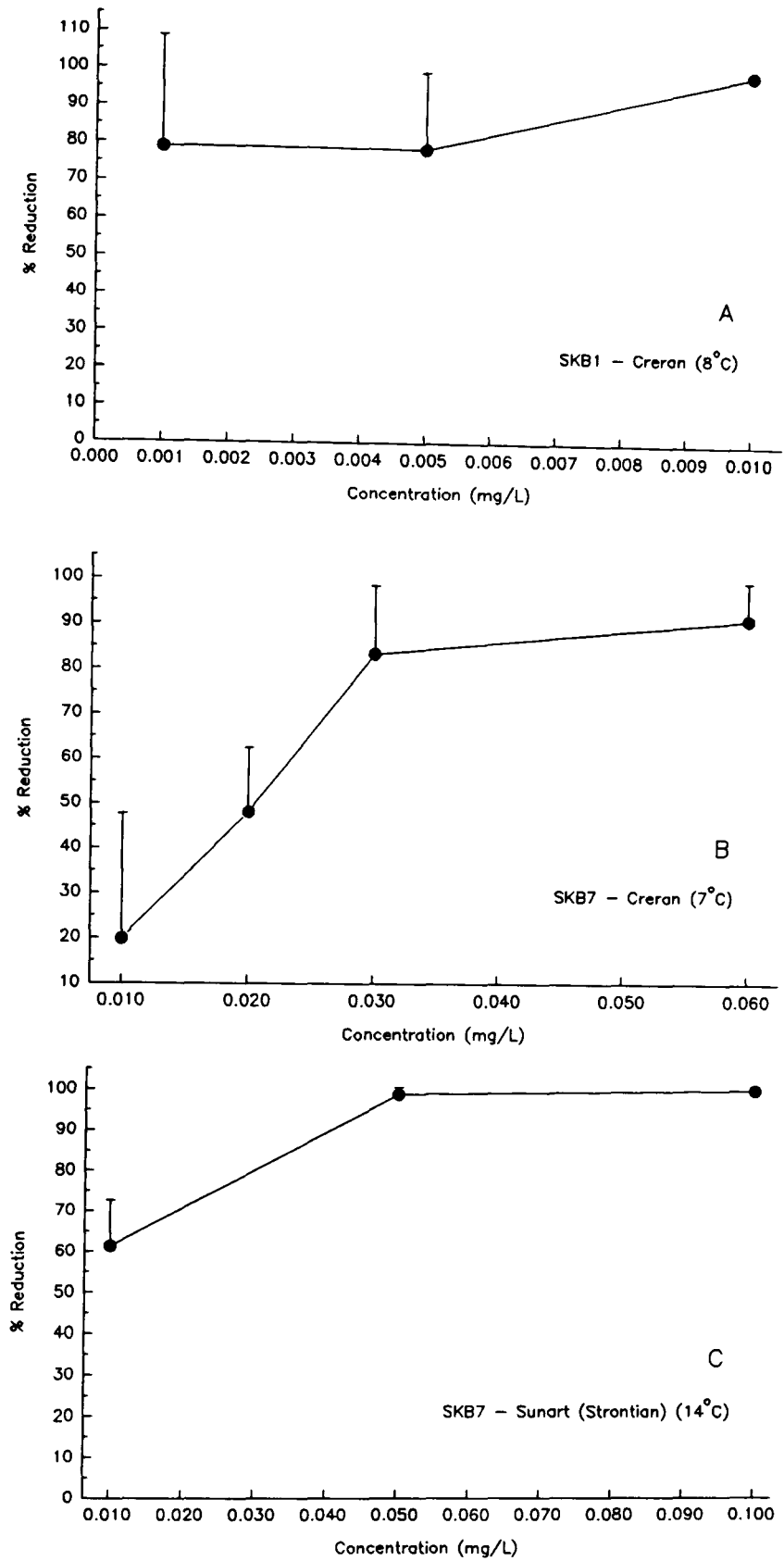| Date	T °C	Sal. (‰)	Fish Wt. ± SD (g)	Lice Source	Conc. mg/L (n fish)	Lice Count $\bar{x} \pm$ SD	Fish Mort.	Comments
SKB1								
14/4/90	8.0	25.0	≈ 450	Creran	P.T. (10)	5.7 ± 4.8	-	low sal. in
					0.0 (10)	3.8 ± 1.6	0	loch week
					0.001 (10)	0.8 ± 1.1	0	preceding
					0.005 (10)	0.8 ± 0.7	0	trial
					0.01 (10)	0.0 ± 0.0	0	
SKB7								
28/8/90	14.0	32.0	154.6 ± 30.8	Sunart-Strontian	0.0 (10)	28.4 ± 12.7	0	
					0.01 (10)	11.0 ± 3.2	0	
					0.05 (10)	0.4 ± 0.5	0	
					0.1 (10)	0.0 ± 0.0	0	
					0.5 (10)	0.0 ± 0.0	9	
					1.0 (10)	0.0 ± 0.0	5	
16/1/91	7.0	31.0	670.0 ± 136.6	Creran	0.0 (12)	9.4 ± 4.4	0	
					0.01 (6)	7.6 ± 2.6	0	
					0.02 (9)	4.9 ± 1.4	0	
					0.03 (9)	1.6 ± 1.4	0	
					0.06 (9)	0.8 ± 0.8	0	

Figure 5.9. Efficacy of compounds SKB1 (A) & SKB7 (B & C) against sea lice from L. Creran (SKB1 & 7) and L. Sunart (Strontian) (SKB7) (bars = SD).

With respect to toxicity to fish, SKB7 was found to have a therapeutic ratio less than ten, with fish tolerating single exposures of 0.1 mg/L but not at higher concentrations tested (Table 5.5).

Temperatures and salinities for the time series trials (Trial 1 & 2) were 7.5°C and 34.5 ‰ respectively. Mean weights of the control fish in the trials were 835.5 g ($\pm$ 151.5 n=10) (Trial 1) and 682.1 g ($\pm$ 197.8 n=20) (Trial 2 - all fish). There were no mortalities in Trial 1. In Trial 2 there were 4 mortalities throughout the course of the experiment. One control fish (runt) died 16 hours post treatment; 1 fish from the resmethrin group died 1 week post treatment and 2 fish died in the SKB7 group, 1 at 24 hours post treatment and 1 one week from treatment.

Results for the efficacy of treatments (% reduction) are given in Figures 5.10a & b. Numbers of lice on control fish did not appear to vary significantly from the beginning to the end of the experiment (T1: $F = 1.53$, $P = 0.14$; T2: $F = 0.14$, $P = 1.00$). For all test compounds optimum efficacy (90%) was not achieved until 8 hours post treatment. The moderate amount of variation found in Trial 1 (Figure 5.10a) is probably due to the sub-sampling technique used. Efficacy following treatment with azamethiphos and resmethrin appeared to reach a steady state after 8 hours with a small proportion of lice remaining on fish 1 week following treatment. The lice counted 1 week post treatment were most likely larval stages which were unaffected by the treatment. Compound SKB7 was different in this respect as, although 90% efficacy was reached 8 hours post treatment, 100% efficacy was achieved one week following treatment, suggesting a possible effect on larval lice.
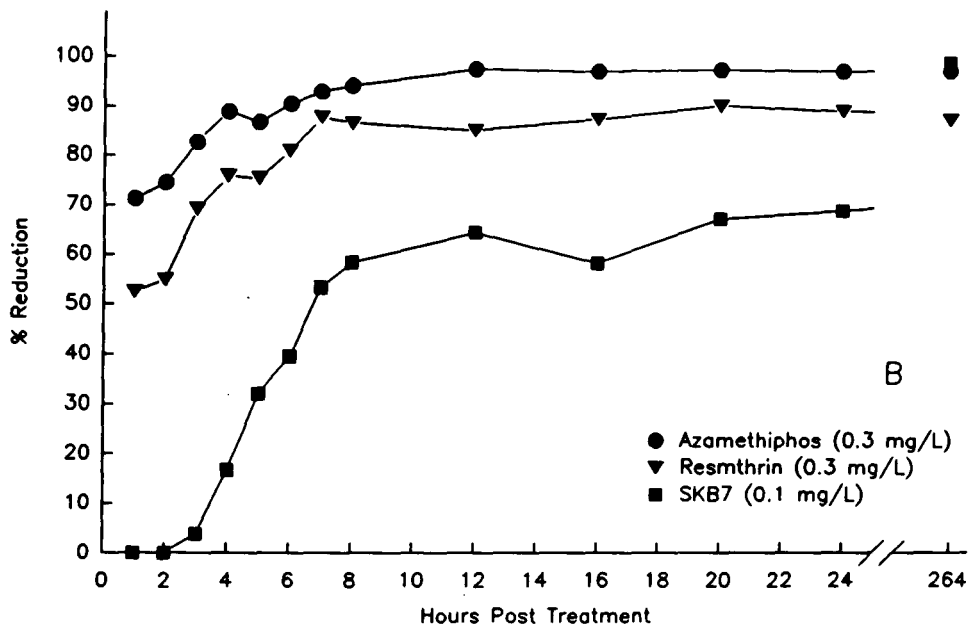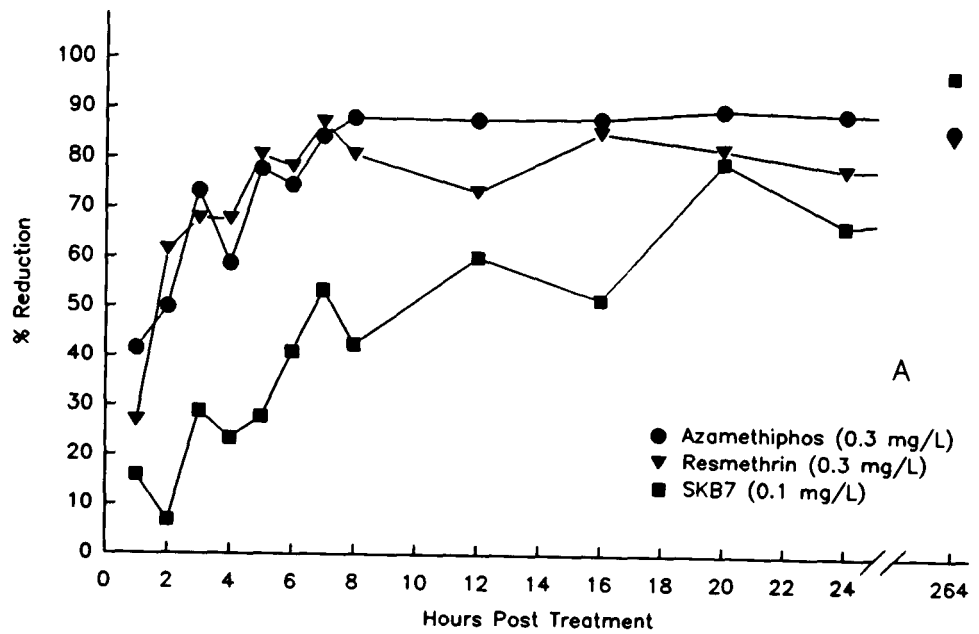
Figure 5.10. Comparative efficacy of azamethiphos, resmethrin & SKB7 against sea lice at various time intervals following exposure (1-hr)
(A - Trial 1, B - Trial 2) (1-h) (bars = SD).

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5.3.2 Field Trials

5.3.2.1 Lice Sensitivity Trials

Results for the sensitivity trials are summarized in Table 5.6. A total of two lice collections was made at Loch Duich. As suggested by the response of lice during routine DDVP treatments, the sensitivity trials revealed that lice from Loch Seaforth were significantly more sensitive to both azamethiphos and DDVP. Creran lice appeared to be marginally less sensitive than lice from Loch Duich. As indicated by the 1 hour post treatment responses, differences between sensitive and non sensitive lice appear to be greater for azamethiphos than for DDVP. However, due to poor survival in control groups, $1+24h$-h LC_{50} values for Loch Seaforth lice could not be calculated and consequently 1-h LC_{50} values should be viewed with caution. The results also showed that the response of lice cannot be accurately assessed immediately after treatment, as indicated by increased mortality during the recovery period following exposure. Lice from Loch Sunart were found to be marginally more sensitive than lice from Lochs Duich and Creran, but were significantly less sensitive than lice from Loch Seaforth.

Table 5.6. 1-h & 1+24-h LC_{50} values (mg/L) for sea lice from different loch systems exposed to azamethiphos and DDVP (values in parenthesis are 95% confidence intervals), ND = not determined.

Site	1-h LC_{50}		1+24-h LC_{50}	
	AZAMETH	DDVP	AZAMETH	DDVP
CRERAN	0.045 (0.28-0.71)	1.10 (0.96-1.25)	0.07 (0.06-0.09)	0.7 (0.56-0.88)
DUICH[1]	0.34 (0.23-0.52)	1.30 (1.14-1.47)	0.18 (0.16-0.21)	1.8 (1.57-2.11)
DUICH[2]	0.40 (0.30-0.54)	1.06 (0.93-1.21)	0.08 (0.07-0.10)	0.61 (0.49-0.76)
SEAFORTH	0.02 (0.01-0.06)	0.09 (0.03-0.29)	ND	ND
SUNART (LAGA)	0.36 (0.11-1.19)	0.39 (0.32-0.49)	0.08 (0.05-0.12)	0.30 (0.22-0.40)

[1] 1st lice collection
[2] 2nd lice collection

5.3.2.2   Efficacy Trials

5.3.2.2.1   Loch Creran

Only two treatments were completed with azamethiphos at Loch Creran, taking place during November and December, 1991. However, a third series of treatments was initiated by the site with DDVP, immediately following the second set of azamethiphos treatments. This latter DDVP treatment was not monitored for efficacy, but a sample was taken from one of the treatment cages to assay brain AChE activity (see below). The average number of fish per net pen was 10,754 with an average weight of 2.37 kg (Table 5.7). Neither temperature nor salinity fluctuated appreciably throughout the course of the trial (Table 5.7). Although more variable, oxygen readings taken inside treated cages did not vary significantly from those taken in the loch immediately adjacent to the cages (Table 5.7). In most instances, O_2 levels were higher inside

tarpaulins during treatment than in the surrounding loch water. A notable exception to the overall high O_2 readings were those taken for the skirt treatments where oxygen diffusors were not used (Table 5.7).

Both sets of the azamethiphos treatments were completed without incident. During the following treatment with DDVP, one of the treatments (cage 7 - included in the azamethiphos trials) was aborted due to a negative reaction from the fish. These fish were sampled for AChE activity which is discussed below.

Mortalities following treatment are summarised in Table 5.7. The numbers of mortalities recorded were not appreciably different than those normally recorded at the site. In addition, 10 mortalities were recorded following the DDVP treatment discussed above.

Results obtained on the efficacy of azamethiphos from the 1st and 2nd treatments are summarized in Figures 5.11 - 5.14. Untreated data of the mean number of lice/fish is summarized in Table 5.8. Results from the first treatment indicated that highest efficacy was achieved at a concentration of 0.2 mg/L, but at this concentration, efficacy 24 hours following treatment was only 47.4% (Figure 5.11). Following the second treatment, better results were obtained with the 3 cages treated at 0.2 mg/L, with an average efficacy of 70% (Figure 5.12). Concentrations of 0.05 and 0.1 mg/L appeared to have little effect and numbers of adult/pre-adult lice increased to levels above pre-treatment levels within a week (Figure 5.11). The skirt treatment (0.2 mg/L) appeared to have no effect following the 1st or 2nd treatment (Figures 5.11 & 5.12).

The compound appeared to have little effect on larval stages of lice at all concentrations tested (Figures 5.13 & 5.14). It was interesting to note that a high number of chalimus stages (probably from recent copepodid settlement) were recorded on the cage which was treated with a skirt rather than full tarpaulin. However, it is likely that in many instances chalimus means were underestimated (Tully 1989).

Table 5.7. Number of fish, weight, amount of azamethiphos used, site specific water quality parameters (temperature, salinity, dissolved oxygen) and mortalities during cage trials at Loch Creran.

Date	Cage	No. Fish	X Weight (kg)	Azameth. added (g)	Target Conc. (mg/L)	T(°C)	Sal. (‰)	Dissolved Oxygen (mg/L)					Fish Mort.
								0	15	30	45	Loch	
1st Treatment													
25/11/91	9	9,349	1.99	158	0.1	10.0	34	9.3	9.6	9.4	9.2	9.1	11
25/11/91	11	9,838	1.86	79	0.05	10.0	34	9.9	10.3	10.2	10.8	9.1	6
25/11/91	13	13,803	1.94	316	0.2	10.0	34	9.9	10.3	10.8	10.5	9.1	7
26/11/91	1	8,449	2.88	316	0.2	10.0	34	8.4	9.1	9.1	9.0	9.0	6
26/11/91	3	14,226	2.67	360 (skirt)	0.2	10.0	34	8.1	8.0	7.6	7.7	9.0	4
26/11/91	5	13,578	2.46	316	0.2	10.0	34	7.9	8.4	8.2	8.3	9.0	16
26/11/91	7	6,433	2.49	316	0.2	10.0	34	9.2	9.9	10.0	10.1	9.0	5
2nd Treatment													
10/12/91	1	8,439	2.18	316	0.2	9.5	34	9.9	10.2	ND	ND	ND	8
10/12/91	3	14,211	2.67	360 (skirt)	0.2	9.5	34	ND	ND	8.7	7.9	ND	13
10/12/91	5	13,550	2.46	316	0.2	9.5	34	ND	ND	10.7	11.9	ND	5
10/12/91	7	6,419	2.49	316	0.2	9.5	34	ND	ND	9.1	9.4	ND	5

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Table 5.8. Efficacy summary for cage trials with azamethiphos at Loch Creran (0 hrs = pre-treatment sample).

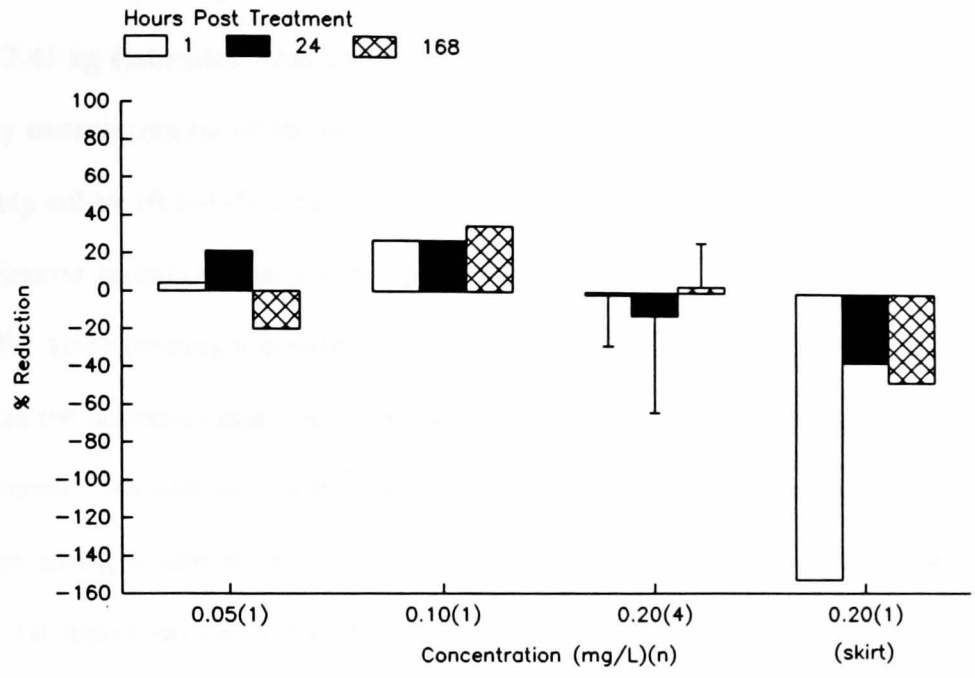|		Mean No. Lice/Fish (n = 10 Fish)							
		Adult & Preadult Lice				Chalimus			
Cage	Dose (mg/L)	Hours Post Treatment							
		0	1	24	168	0	1	24	16
1st Treatment									
1	0.2	18.4	15.8	17.1	19.1	5.1	5.1	5.7	3.2
3	0.2-skirt	8.2	10.6	9.8	14.4	1.9	4.8	2.6	2.8
5	0.2	10.0	5.6	4.1	7.5	2.4	1.7	2.1	2.1
7	0.2	8.3	6.3	3.0	8.6	3.4	3.1	1.9	4.1
9	0.1	6.2	5.8	4.5	8.7	2.6	1.9	1.9	1.7
11	0.05	8.1	6.2	6.5	12.1	2.4	2.3	1.9	2.9
13	0.2	12.9	7.4	5.2	8.8	1.8	2.6	3.5	2.1
2nd Treatment									
1	0.2	30.0	25.0	10.3	9.6	2.7	2.3	2.6	2.7
3	0.2-skirt	18.3	18.4	17.5	18.2	2.6	2.0	2.1	1.9
5	0.2	14.5	5.1	2.6	3.2	2.6	1.0	1.4	1.8
7	0.2	10.6	10.0	4.0	3.8	2.0	1.8	2.8	2.8
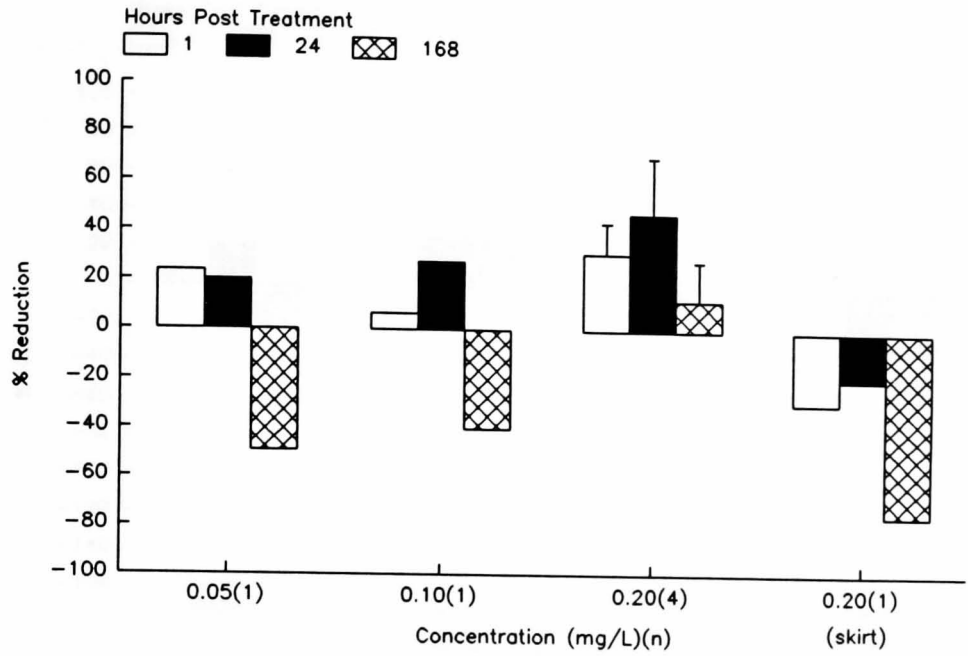
Figure 5.11. Efficacy of azamethiphos against adult/preadult lice following the 1st treatment at Loch Creran (n = no. cages sampled, bars = SD).
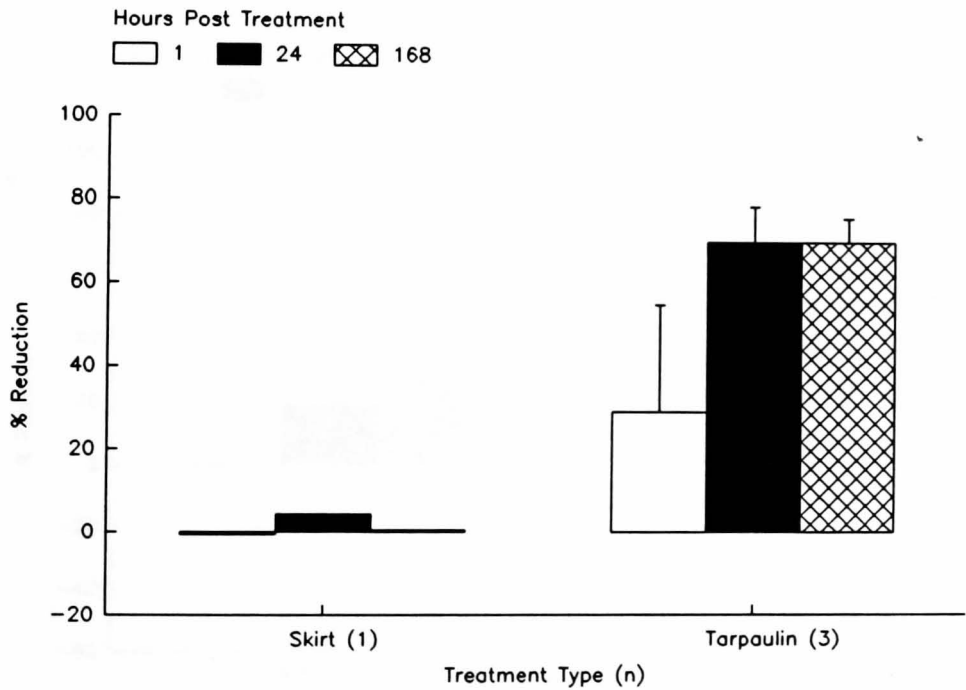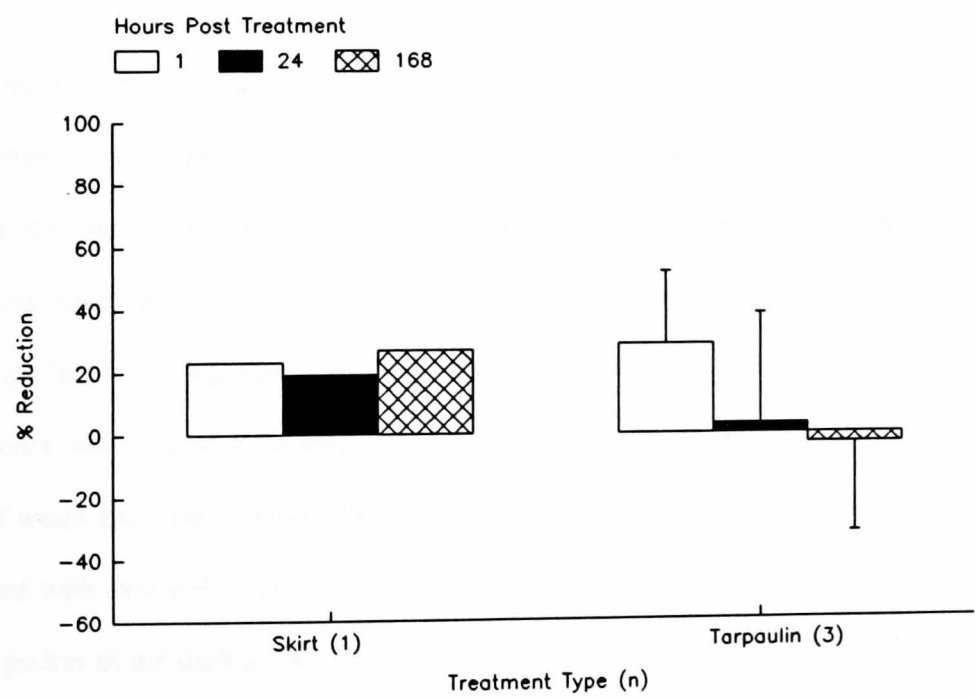

Figure 5.12. Efficacy of azamethiphos (0.2 mg/L) against adult/preadult lice following the 2nd treatment at Loch Creran (n = no. cages sampled, bars = SD).

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Figure 5.13. Efficacy of azamethiphos against larval lice following the 1st treatment at Loch Creran (n = no. cages sampled, bars = SD).

Figure 5.14. Efficacy of azamethiphos (0.2 mg/L) against larval lice following the 2nd treatment at Loch Creran (n = no. cages sampled, bars = SD).

5.3.2.2.2   Loch Duich

A total of three treatments was completed at Loch Duich from November 1991 to February 1992. The average number of fish per cage was 4,659 with an average weight of 2.41 kg (estimated from cages 5,6,7 & 8), neither of which changed appreciably over the course of the study (Table 5.9). Temperatures for these trials were notably colder (6.5-9.0°C) than for the Loch Creran trials (Table 5.9). The site also experienced periods of low salinity, probably the result of heavy rain water run-off (Table 5.9). Unfortunately dissolved oxygen readings could only be made for the 1st treatment as the necessary equipment was not available on site during the second and third treatments. As with the Loch Creran treatments, O_2 readings tended to be higher in the cages during treatment than in the surrounding loch water; in one of the cages (cage 12 - 1st treatment) the O_2 readings were particularly high (Table 5.9). Prior to treatment, the diffusor in this cage malfunctioned and was replaced with two diffusors, probably accounting for the high O_2 readings.

Although most of the treatments carried out at this site were without incidence, some difficulties were encountered. A primary constraint to treatment was the strong tidal currents in the loch. Treatments could only made during a short window in the tide cycle, during slack tide (approx 1/month). During the first treatment, water in one of the cages (9) had to be supplemented with water pumped in from the loch via an outboard boat motor as wind became trapped under the tarpaulin thereby decreasing the volume of water enclosed. During the third treatment, similar problems were encountered with two additional cages (9 & 10). These cases differed, as wind slowly formed a pocket of air during the course of treatment. Since the fish in the first cage (9) did not appear to be stressed the treatment was allowed to continue for 1 hour.

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However, fish in the second cage (10) were showing signs of stress and therefore the treatment was stopped after 40 minutes and the tarpaulin dropped.

There were few mortalities following most of the treatments (Table 5.9). However, there were notable mortalities in one cage (9) where 107 fish died (= 1.7%) following the first treatment. It was impractical to sample the fish for either histology or AChE levels as the fish were discovered during the routine dead count 48 hours from treatment (as described above). Furthermore, 3 days following the discovery of the dead fish, fish in cage 9 tested positive for *Aeromonas salmonicida*, which had appeared elsewhere on the site a week earlier and which was also the site's first case of the disease. Thus it was not known whether the fish died as a result of the bacterial infection or had contracted the infection due to the stress of treatment. Alternatively, both the presence of *A. salmonicida* and the stress from treatment could have contributed to the mortalities. Interestingly, an adjacent cage (11) was also sampled for bacteriology and tested negative for *A. salmonicida*.

Results for the efficacy of treatment are summarized in Figures 5.15 - 5.18 (see Table 5.10 for untreated lice data). The results from the 1st set of treatments indicated that 0.2 mg/L gave the best efficacy, with an overall mean of 71.6% and range of 41.9 to 96.6% (24 hours from treatment) (Figure 5.15). The treatment at 0.05 mg/L was poorly efficacious as indicated by the 24 hour post treatment results (lice could not be estimated 1 week following treatment as the fish had been moved to another location on the site). Similarly, 0.1 mg/L appeared to have little effect (Figure 5.15). Concentrations of 1.0 mg/L DDVP were substantially more efficacious than 0.1 mg/L azamethiphos, but not as effective as 0.2 mg/L (azamethiphos) (Figure 5.15).

A consistent pattern was evident in reductions of adult and preadult lice over the course of the three treatments at 0.2 mg/L (Figure 5.16). Small reductions in lice were seen at the end of the treatment (1 h) and ranged from as low as 1.7% to as high as 69.2%. These reductions were followed with further reductions 24 hours following treatment (range of 41.9% to 96.6% efficacy) which were followed by increases in overall lice numbers 1 week from treatment (Figure 5.16, Table 5.10).

Consistent with the findings at Loch Creran, there appeared to be little effect against larval stages (Figures 5.17 & 5.18). Some reductions were seen but the mean numbers counted varied considerably from cage to cage (Table 5.10).

Table 5.9. Number of fish, weight, amount of azamethiphos used, site specific water quality parameters (temperature, salinity, dissolved oxygen) and mortalities during cage trials at Loch Duich.

Date	Cage	No. Fish	X Weight (kg)	Azameth. used (g)	Target Conc. (mg/L)	T (°C)	Sal. (‰)	Dissolved Oxygen (mg/L)					Fish Mort.
								0	15	30	45	Loch	
1st Treatment													
28/11/91	9	6,155	2.09	172	0.2	9.0	28	11.8	12.5	12.6	12.6	13.0	107
28/11/91	10	7,168	2.15	86	0.1	9.0	28	12.6	12.6	12.6	13.0	13.0	0
28/11/91	11	4,435	ND	700 ml[*]	1.0[+]	9.0	28	12.7	15.1	13.4	13.3	13.0	0
28/11/91	12	1,885	ND	43	0.05	9.0	28	14.7	16.7	16.5	18.7	13.0	0
29/11/91	5	4,452	1.8	172	0.2	9.0	29	11.5	12.8	13.0	13.4	13.0	0
29/11/91	6	5,479	2.2	172	0.2	9.0	29	10.9	12.2	12.9	12.8	13.0	1
29/11/91	7	4,583	2.31	172	0.2	9.0	29	12.3	12.2	12.8	12.6	13.0	0
29/11/91	8	5,988	2.30	172	0.2	9.0	29	12.2	13.0	15.1	15.0	13.0	3
2nd Treatment													
15/1/92	5	4,404	1.86	172	0.2	6.5	19	ND	ND	ND	ND	ND	1
15/1/92	6	4,467	1.96	172	0.2	6.5	19	ND	ND	ND	ND	ND	3
15/1/92	7	4,575	2.74	172	0.2	6.5	19	ND	ND	ND	ND	ND	0
15/1/92	8	4,308	2.52	172	0.2	6.5	19	ND	ND	ND	ND	ND	0
3rd Treatment													
25/2/92	7	4,573	2.98	172	0.2	7.5	24	ND	ND	ND	ND	ND	0
25/2/92	8	4,297	2.76	172	0.2	7.5	24	ND	ND	ND	ND	ND	0
25/2/92	9[N]	4,334	2.75	172	0.2	7.5	24	ND	ND	ND	ND	ND	0
25/2/92	10[N]	4,452	2.80	172	0.2	7.5	24	ND	ND	ND	ND	ND	0

[*] AQUAGARD[*], [+] mg/L DDVP, [N] new cage

Table 5.10. Efficacy summary (untreated data) for cage trials with azamethiphos at Loch Creran (0 hrs = pre-treatment sample), ND = not determined.

Cage	Dose (mg/L)	Adults & Preadults				Chalimus			
		Hours Post Treatment							
		0	1	24	168	0	1	24	168
1st Treatment									
5	0.2	20.6	14.0	0.7	4.3	3.8	3.7	2.4	1.3
6	0.2	14.7	12.9	6.7	10.5	2.0	3.1	3.0	1.8
7	0.2	17.9	17.6	4.9	8.0	3.0	3.8	2.2	2.5
8	0.2	14.8	10.5	8.6	15.5	2.9	2.1	1.7	3.6
9	0.2	17.6	6.4	1.3	5.0	3.0	2.4	2.9	1.8
10	0.1	22.2	20.1	19.7	18.4	2.8	3.8	3.2	1.1
11	1.0 DDVP	26.9	21.2	11.8	26.1	5.9	7.0	3.3	3.3
12	0.05	14.8	10.9	10.0	ND	2.9	2.5	3.5	ND
2nd Treatment									
5	0.2	51.8	34.8	10.8	28.9	9.0	6.6	6.6	6.5
6	0.2	38.6	31.4	11.5	22.2	5.3	6.1	5.2	3.2
7	0.2	64.3	19.8	9.5	14.5	15.2	4.1	6.0	8.6
8	0.2	42.8	30.2	13.8	18.8	8.3	6.4	4.7	5.1
3rd Treatment									
7	0.2	34.5	27.6	7.6	16.0	6.6	6.3	4.5	8.5
8	0.2	36.4	27.6	20.1	24.9	5.9	8.2	6.6	7.0
9^N	0.2	34.6	22.8	10.9	15.1	6.9	5.8	6.9	6.9
10^N	0.2	32.4	17.2	15.9	21.3	10.9	7.6	6.2	7.2

Mean No. Lice/Fish (n = 10 Fish)

[N] new cage, * cage not sampled
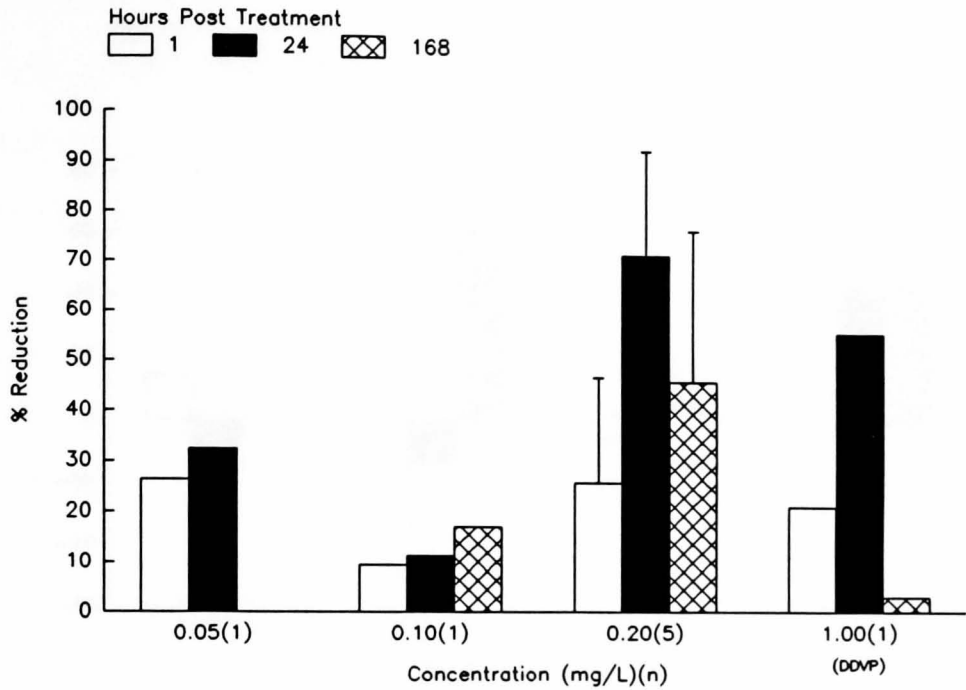
Figure 5.15. Efficacy of azamethiphos (and DDVP) against adult/preadult lice following the 1st treatment at Loch Duich (n = no. cages sampled, bars = SD).
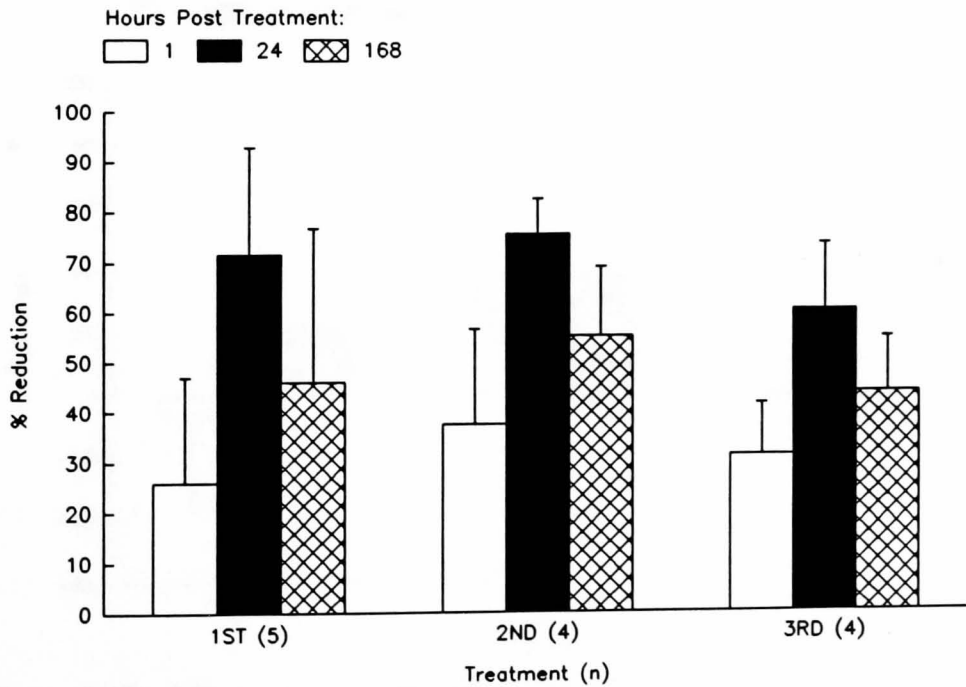
Figure 5.16. Efficacy of azamethiphos against adult/preadult lice following the 1st, 2nd & 3rd treatments at Loch Duich (n = no. of cages sampled, bars = SD).
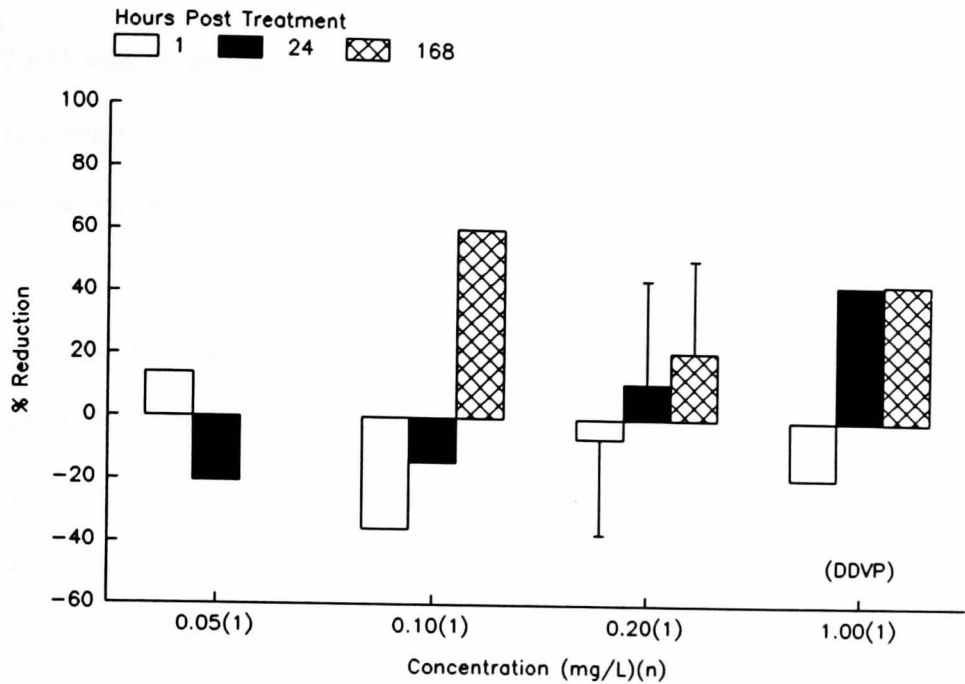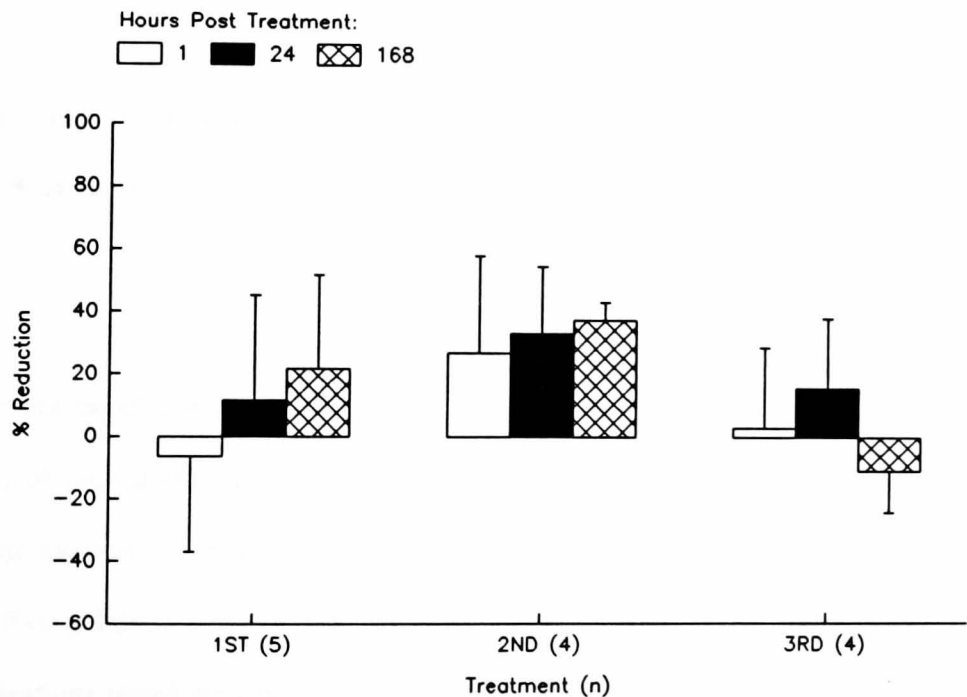
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Figure 5.17. Efficacy of azamethiphos (and DDVP) against larval lice following the 1st treatment at Loch Duich (n = no. cages sampled, bars = SD).

Figure 5.18. Efficacy of azamethiphos (0.2 mg/L) against larval lice following the 1st, 2nd & 3rd treatments at Loch Duich (n = no. cages sampled, bars = SD).

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5.3.2.2.3   Loch Seaforth

A total of two treatments was conducted at the Loch Seaforth site, with the first in
December 1991 and the second in January 1992. The number of fish in the cages
averaged 7,673 with an average weight of 0.77 kg (Table 5.11). However, as seen in
Table 5.11, average weights appeared to vary somewhat from cage to cage and from
the first and second treatment.

Temperatures were 8.5 and 7.8°C for the 1st and 2nd treatment respectively (Table
5.11). Salinities were 30‰ and 34‰ for the 1st and 2nd treatments respectively
(Table 5.11). Dissolved oxygen could not be measured during the 1st treatment, due to
technical difficulties, but was measured for all four of the second treatments.
Consistent with the findings for the treatments at Lochs Creran and Duich, O_2 was
generally higher in cages during treatment than in the loch water surrounding cages
(Table 5.11).

There were only 3 mortalities recorded throughout the trial (cages checked 24 hours
following treatment). All three mortalities were attributed to predator damage (Table
5.11).

Unlike the lice populations at Lochs Creran & Duich, which were comprised almost
exclusively of *Lepeophtheirus salmonis*, lice at Loch Seaforth were of both *L. salmonis*
and *Caligus elongatus*. Efficacy for the two treatments is summarized in Figures
5.19 - 5.30 (see Tables 5.12a & b for untreated data). Following the first treatment,
all concentrations tested demonstrated efficacies greater than 85% in removing adult
and preadult lice following treatment with negligible differences between the two

species of lice (Figures 5.19, 5.21 & 5.22). Differences between the efficacies of treatments at 0.1 and 0.2 mg/L were marginal with a slightly delayed response seen in the 0.05 mg/L treatment (Figures 5.19, 5.21, 5.22, 5.29 & 5.30). Although there appeared to be negligible differences between the response of the two species of lice to azamethiphos, *C. elongatus* appear to be somewhat more sensitive than *L. salmonis* following treatment with DDVP at 1.0 mg/L (Figure 5.21 & 5.22). Although 90% efficacious, DDVP (1.0 mg/L) did not perform as well as azamethiphos at any of the concentrations tested (0.05, 0.1 & 0.2 mg/L) 24 hours from treatment (Figure 5.19, 5.21 & 5.22). Similar results between the two treatments were obtained with azamethiphos at 0.1 and 0.2 mg/L treatment (Figures 5.23, 2.25 & 5.26).

Consistent with the findings at the other sites studied, there was little if any effect on the numbers of larval lice. Following the first treatment, chalimus numbers appeared to fluctuate between sampling points with increases in numbers of chalimus stages observed at 1 and 24 hours following treatment and decreases 1 week following treatment (Figure 5.20). In addition, no differences were seen between *C. elongatus* and *L. salmonis* responses (Figures 5.20, 5.24, 5.27 & 5.28). Larval lice counts during the second treatment indicated a large settlement of chalimus on fish between sampling periods, largely attributed to *L. salmonis* larvae with 5 fold increases in larval lice numbers (Figures 5.20, 5.22, 5.27 & 5.28).

Table 5.11. Number of fish, weight, amount of azamethiphos used, site specific water quality parameters (temperature, salinity, dissolved oxygen) and mortalities during cage trials at Loch Seaforth (ND = not determined).

Date	Cage	No. Fish	X Weight (kg)	Azameth. used (g)	Target Conc (mg/L)	Temp. (°C)	Sal. (‰)	Dissolved Oxygen (mg/L)					Fish Mort.
								0	15	30	45	Loch	
1st Treatment													
11/12/91	1	7,840	0.75	43	0.05	8.5	30	ND	ND	ND	ND	ND	0
11/12/91	2	8,302	0.73	86	0.1	8.5	30	ND	ND	ND	ND	ND	0
11/12/91	3	8,210	0.80	172	0.2	8.5	30	ND	ND	ND	ND	ND	1[P]
11/12/91	4	8,382	0.82	600 mL*	1.0[+]	8.5	30	ND	ND	ND	ND	ND	0
12/12/91	5	8,388	0.79	86	0.1	8.5	30	ND	ND	ND	ND	ND	0
12/12/91	6	8,452	0.74	86	0.1	8.5	30	ND	ND	ND	ND	ND	0
12/12/91	7	6,385	0.62	86	0.1	8.5	30	ND	ND	ND	ND	ND	0
12/12/91	8	6,829	0.50	86	0.2	8.5	30	ND	ND	ND	ND	ND	2[P]
2nd Treatment													
30/1/92	5	8,261	1.17	86	0.1	7.8	34	12.7	10.4	9.6	9.6	8.5	0
30/1/92	6	8,325	1.13	86	0.1	7.8	34	8.9	8.7	8.8	9.3	8.5	0
30/1/92	7	6,220	0.90	86	0.1	7.8	34	8.0	8.3	11.2	12.5	8.3	0
30/1/92	8	6,656	0.79	172	0.2	7.8	34	9.3	8.3	9.7	ND	8.5	0

* AQUAGARD®, + mg/L DDVP, [P] predator damage

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Table 5.12a. Efficacy summary (untreated data), for adult and pre-adult lice, for cage trials with azamethiphos at Loch Seaforth (0 hrs = pre-treatment sample).

		Mean No. Lice/Fish (n = 10 Fish)											
		Combined				L. salmonis				C. elongatus			
Cage	Dose (mg/L)					Hours Post Treatment							
		0	1	24	168	0	1	24	168	0	1	24	168
1st Treatment													
1	0.05	25.5	3.4	1.4	0.3	11.3	1.2	0.3	0.2	14.2	2.3	1.1	0.2
2	0.1	28.1	2.8	0.9	1.0	9.6	0.5	0.1	0.6	18.5	2.3	0.8	0.4
3	0.2	21.3	1.5	1.8	0.4	7.5	0.5	0.2	0.2	13.8	1.0	1.6	0.2
4	1.0 DDVP	23.0	1.7	2.2	1.7	6.0	0.7	0.7	0.8	17.0	1.0	1.5	0.9
5	0.1	22.3	0.8	0.4	0.6	5.1	0.4	0.0	0.2	17.2	0.4	0.4	0.4
6	0.1	25.4	0.8	0.4	0.3	8.7	0.1	0.2	0.2	16.6	0.7	0.2	0.1
7	0.1	16.5	0.4	0.4	0.0	7.2	0.3	0.0	0.0	9.4	0.1	0.4	0.0
8	0.2	16.5	0.0	0.3	0.3	6.7	0.0	0.0	0.0	9.8	0.0	0.3	0.3
2nd Treatment													
5	0.1	27.8	1.0	0.3	0.2	14.1	0.6	0.1	0.1	13.7	0.4	0.2	0.1
6	0.1	25.5	1.3	0.3	1.0	13.0	0.9	0.1	0.1	12.5	0.4	0.2	0.9
7	0.1	27.8	1.1	0.3	0.5	14.3	0.7	0.2	0.4	13.5	0.4	0.1	0.1
8	0.2	26.2	0.9	0.2	1.0	12.3	0.6	0.2	0.1	14.0	0.3	0.0	0.9

Table 5.12b. Efficacy summary (untreated data), for larval lice (chalimus), for cage trials with azamethiphos at Loch Seaforth (0 hrs = pre-treatment sample) (ND = not determined).

		Mean No. Lice/Fish (n = 10 Fish)											
		Combined				*L. salmonis*				*C. elongatus*			
Cage	Dose (mg/L)					Hours Post Treatment							
		0	1	24	168	0	1	24	168	0	1	24	168
1st Treatment													
1	0.05	5.7	9.0	11.8	4.7	ND	ND	ND	0.5	ND	ND	ND	4.2
2	0.1	6.1	10.7	14.2	6.4	ND	ND	ND	1.7	ND	ND	ND	4.7
3	0.2	5.5	8.3	9.8	4.7	ND	ND	ND	0.5	ND	ND	ND	4.2
4	1.0 DDVP	5.7	8.4	8.5	4.7	ND	ND	ND	1.0	ND	ND	ND	3.7
5	0.1	5.3	5.8	5.0	4.5	0.9	0.7	0.2	0.6	4.4	5.1	4.8	3.9
6	0.1	5.7	7.3	4.1	4.2	1.0	0.7	0.4	0.3	4.7	6.6	3.6	3.9
7	0.1	4.7	1.9	4.2	1.9	1.1	0.6	0.5	0.5	3.6	1.3	3.6	1.4
8	0.2	4.2	3.9	3.0	2.1	0.6	0.3	0.5	0.3	3.6	3.8	2.5	1.8
2nd Treatment													
5	0.1	2.1	4.4	4.7	3.8	0.3	1.9	1.0	1.8	1.7	2.8	3.4	2.0
6	0.1	2.6	4.4	4.9	4.5	1.2	1.4	0.7	2.3	1.4	3.0	4.2	2.2
7	0.1	3.3	3.9	5.9	4.3	1.9	1.2	1.1	2.2	1.4	2.7	4.8	2.1
8	0.2	3.7	4.1	4.4	4.7	2.1	1.2	0.8	2.6	1.6	2.9	3.6	2.1
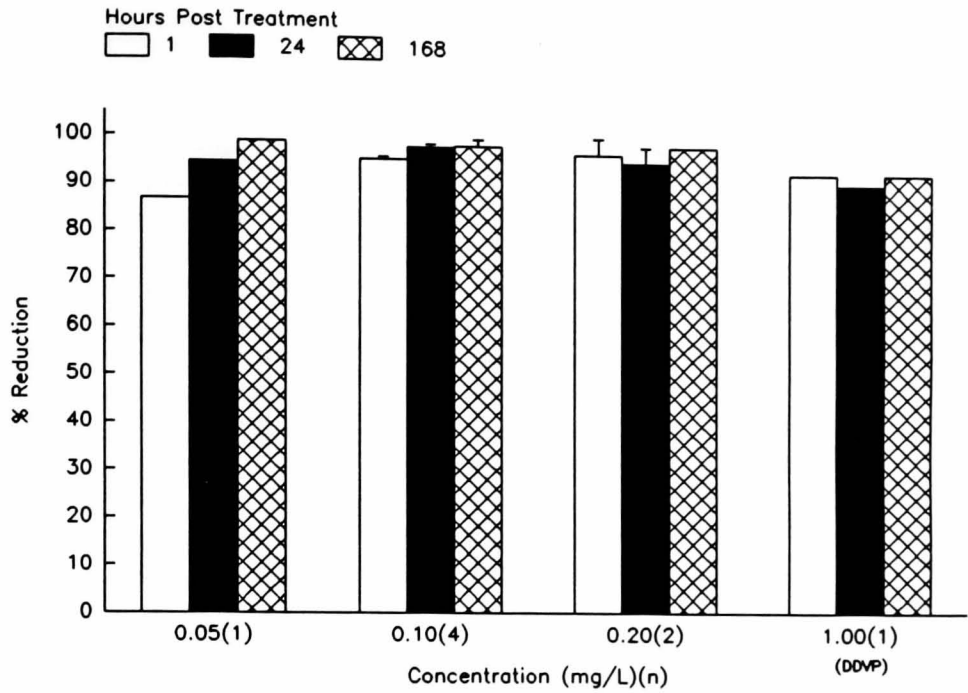
Figure 5.19. Efficacy of azamethiphos (and DDVP) against adult/preadult *L. salmonis* & *C. elongatus* following the 1st treatment at Loch Seaforth (n= no. cages sampled, bars = SD.).
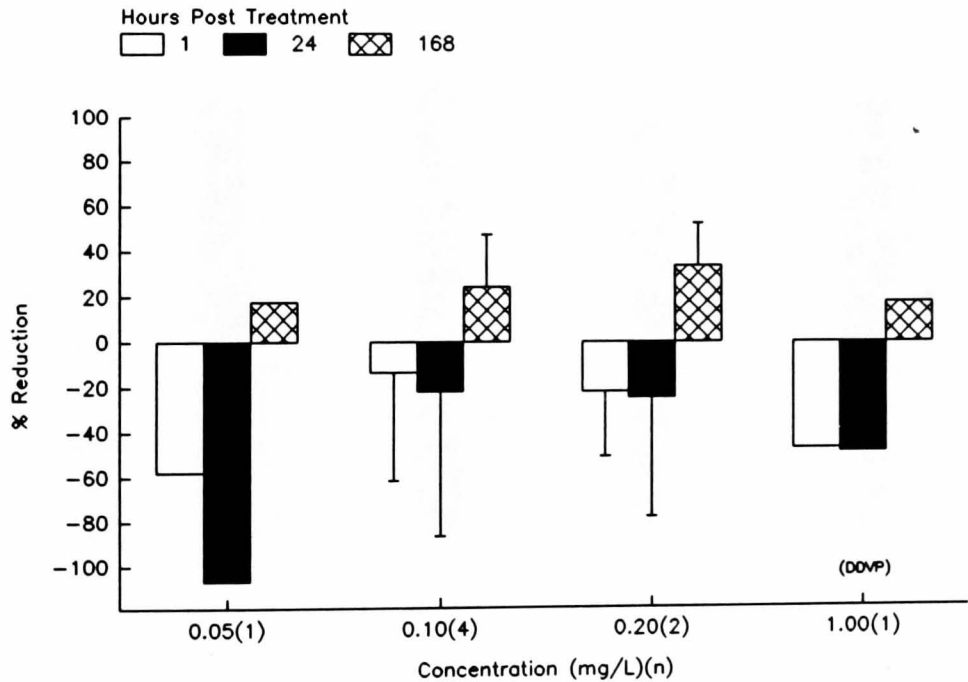
Figure 5.20. Efficacy of azamethiphos (and DDVP) against larval *L. salmonis* & *C. elongatus* following the 1st treatment at Loch Seaforth (n = no. cages sampled, bars = SD).
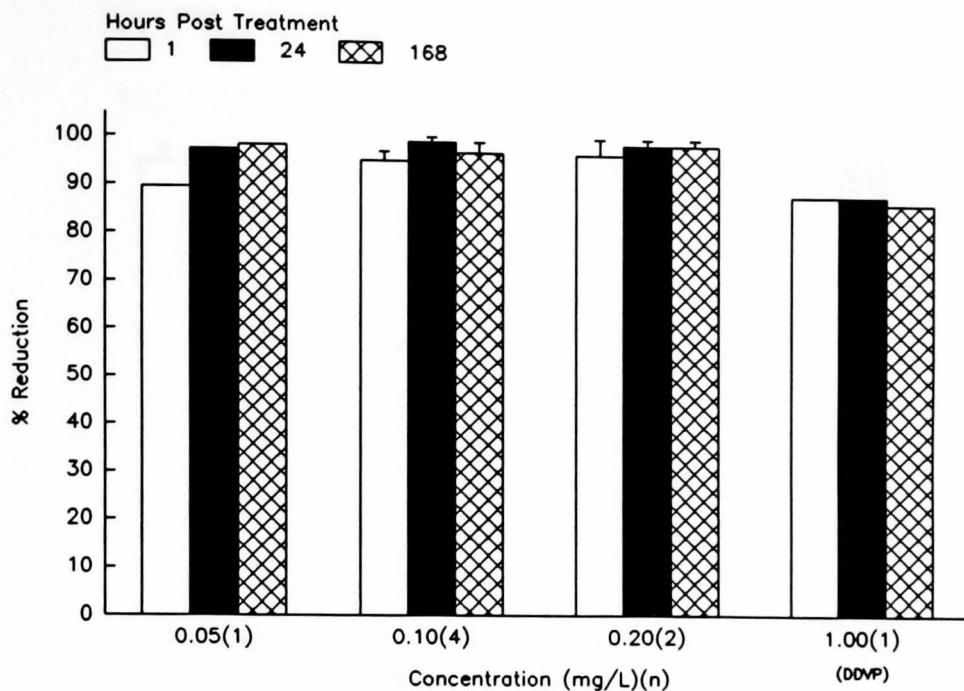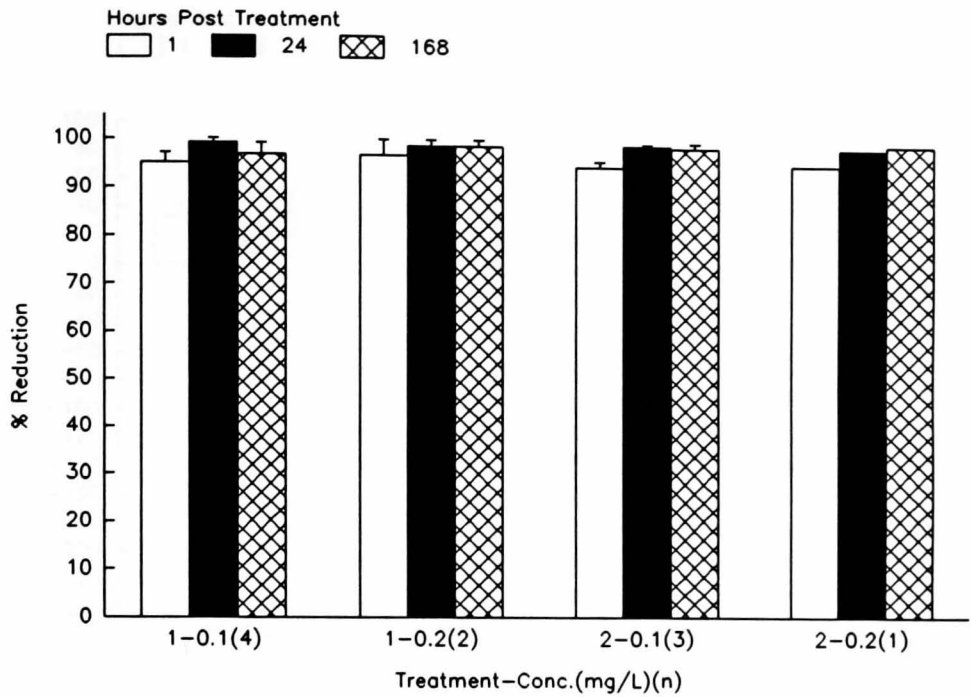
Figure 5.21. Efficacy of azamethiphos (and DDVP) against adult/preadult *L. salmonis* following the 1st treatment at Loch Seaforth (n = no. cages sampled, bars = SD).
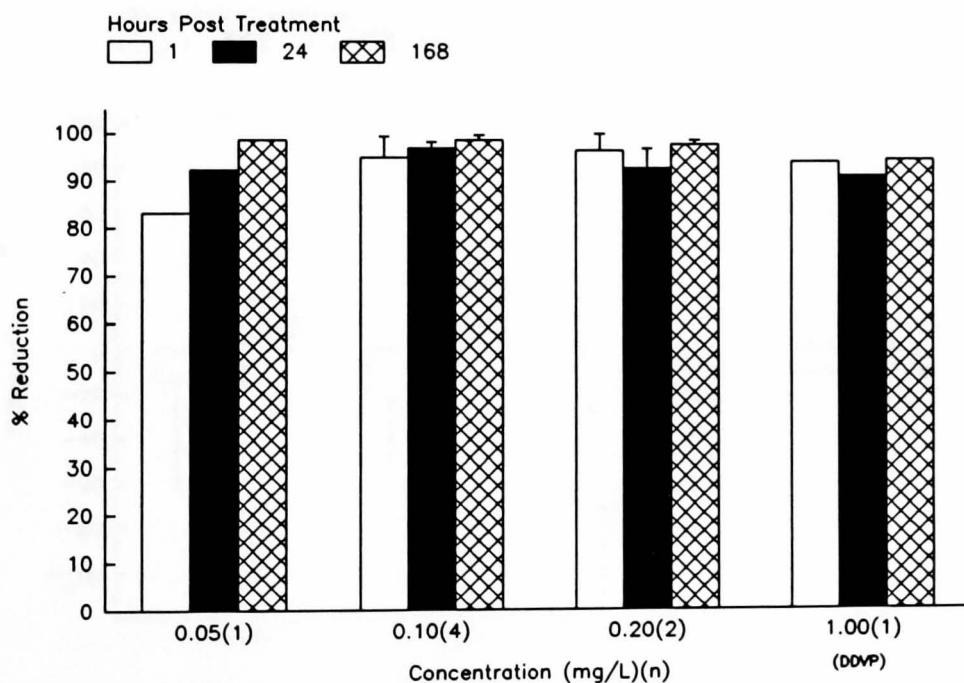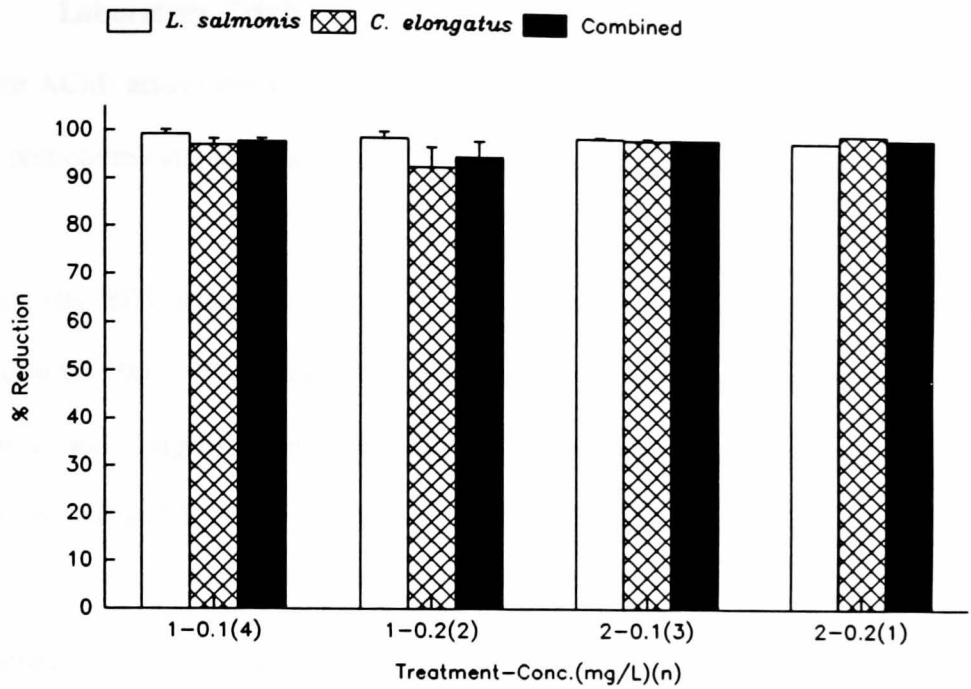
Figure 5.22. Efficacy of azamethiphos against adult/preadult *C. elongatus* following the 1st treatment at Loch Seaforth (n = no. cages sampled, bars = SD).
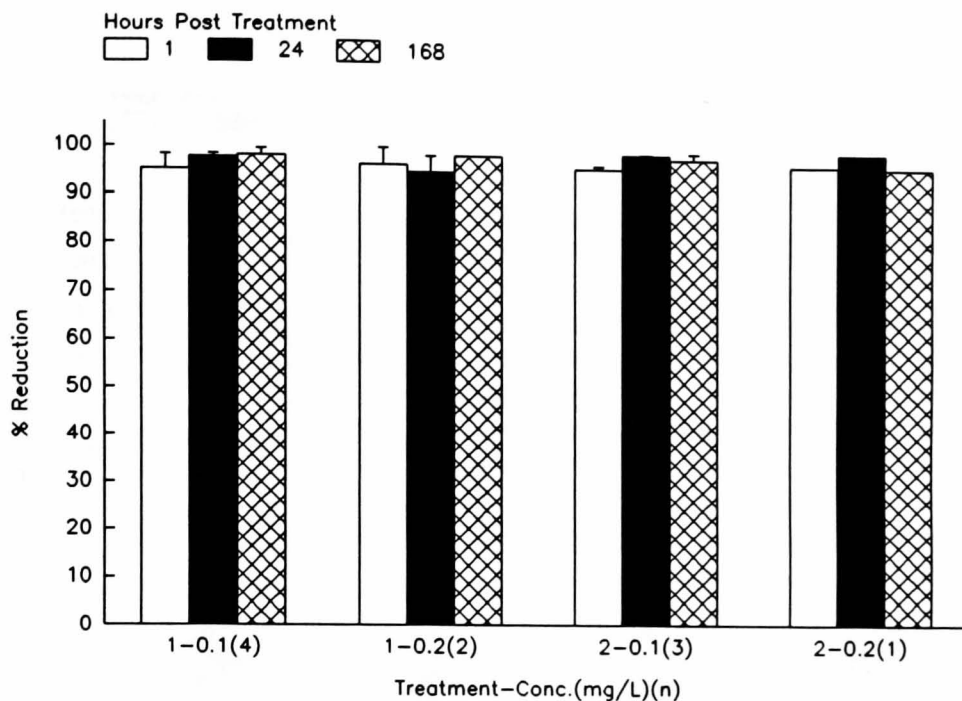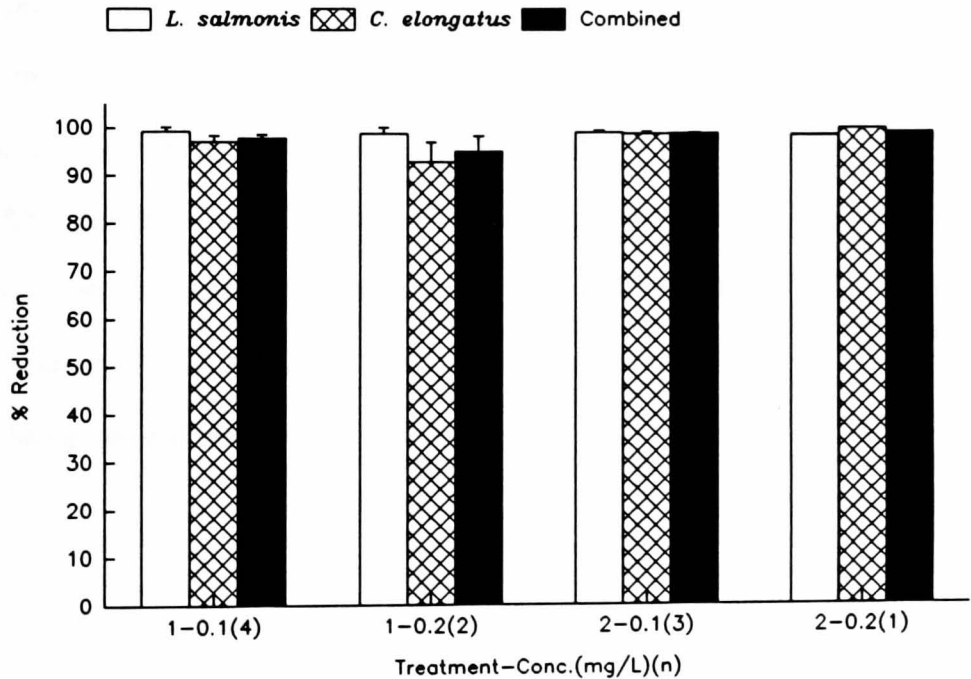
Figure 5.23. Efficacy of azamethiphos against adult/preadult *L. salmonis* & *C. elongatus* following the 1st & 2nd treatment at Loch Seaforth (n = no. cages sampled, bars = SD).
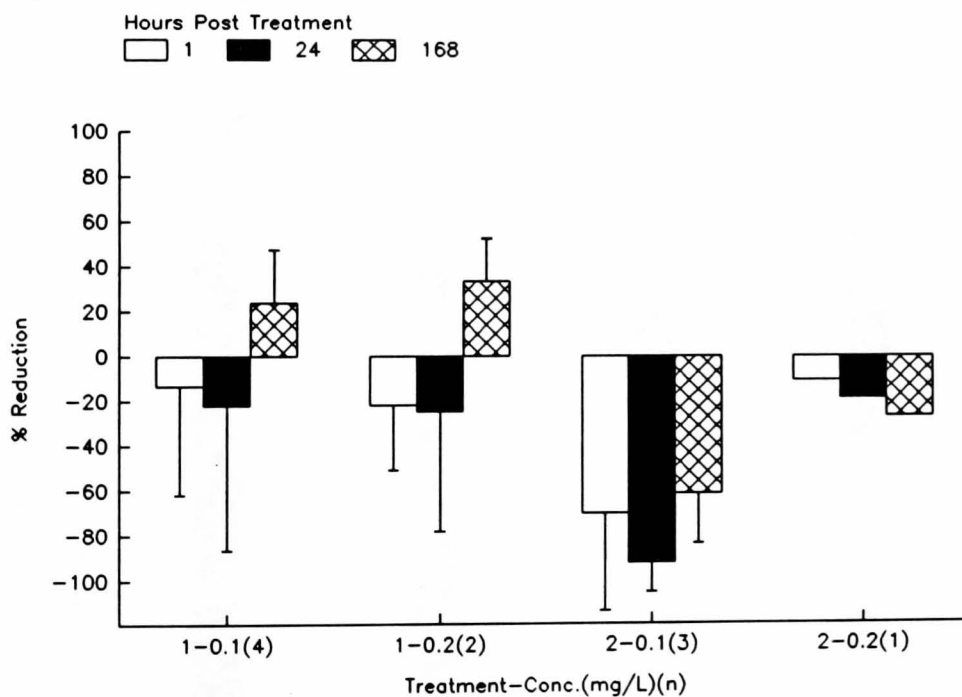
Figure 5.24. Efficacy of azamethiphos against larval *L. salmonis* & *C. elongatus* following the 1st treatment at Loch Seaforth (n = no. cages sampled, bars = SD).

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Figure 5.25. Efficacy of azamethiphos against adult/preadult *L. salmonis* following the 1st and 2nd treatment at Loch Seaforth (n = no. cages sampled, bars = SD).
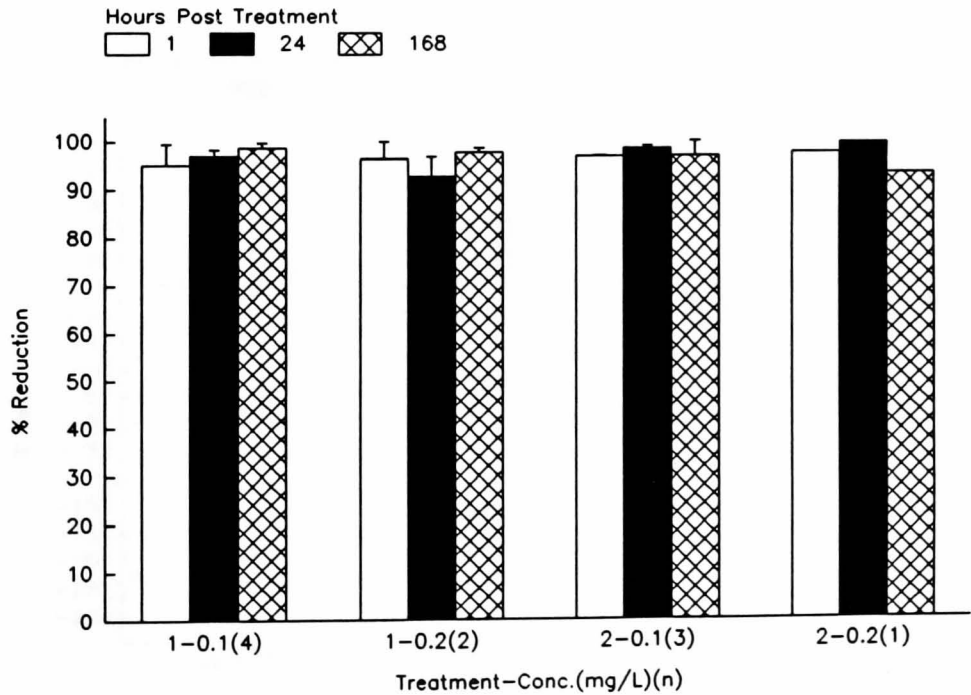
Figure 5.26. Efficacy of azamethiphos against adult/preadult *C. elongatus* following the 1st & 2nd treatment at Loch Seaforth (n = no. cages sampled, bars = SD).

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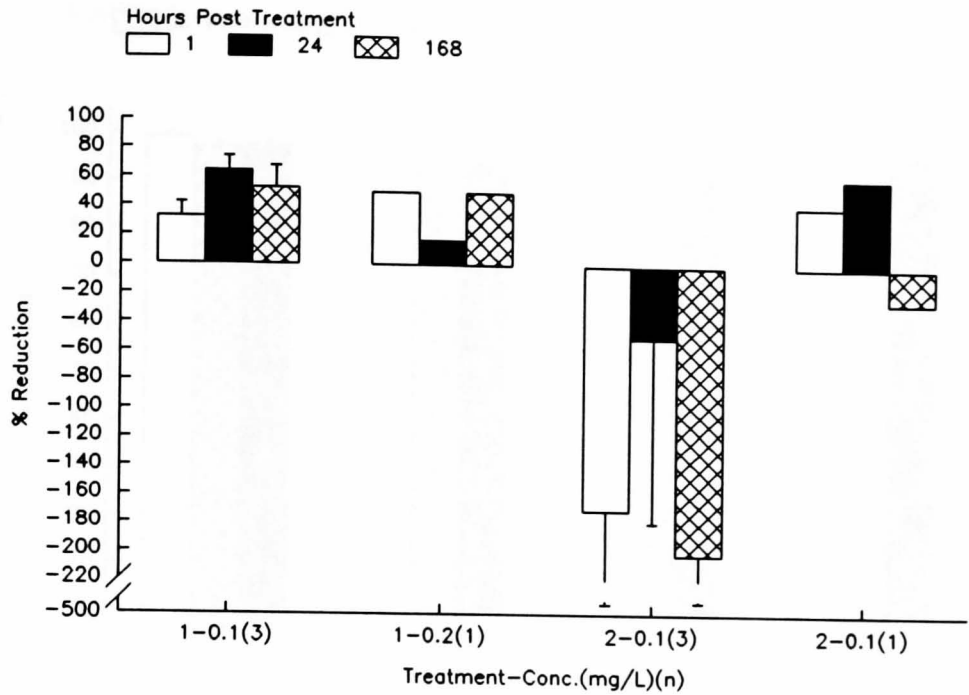
Figure 5.27. Efficacy of azamethiphos against larval *L. salmonis* following the 1st & 2nd treatment at Loch Seaforth (n= no. cages sampled, bars = SD).
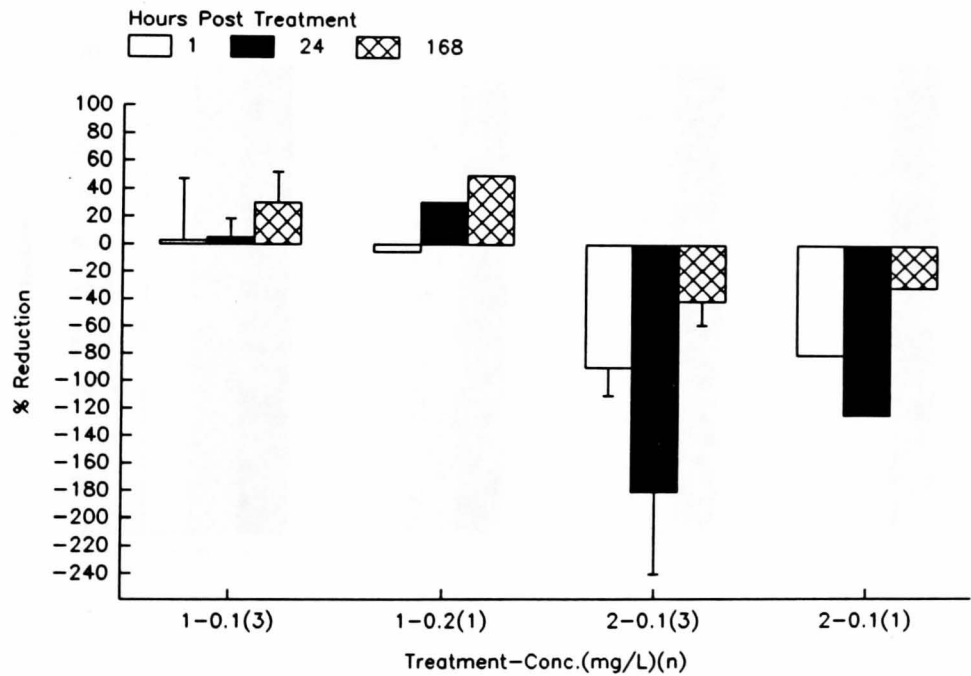
Figure 5.28. Efficacy of azamethiphos against larval *C. elongatus* following the 1st & 2nd treatment at Loch Seaforth (n = no. cages sampled, bars = SD).

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Figure 5.29. Efficacy of azamethiphos against adult/preadult lice 24 hours following treatment at Loch Seaforth (n = no. cages sampled, bars = SD).

Figure 5.30. Efficacy of azamethiphos against larval lice 24 hours following treatment at Loch Seaforth (n = no. cages sampled, bars = SD).

5.3.3 Acetylcholinesterase Assays

5.3.3.1   Laboratory Trials

Data for the AChE assays are summarized in Tables 5.13 and 5.14. Transformed data (% AChE reductions) are summarized in Figures 5.31 - 5.38.

Compound CH650003 was tested at two concentrations, 1.0 and 2.0 mg/L, and both were found to be 100% efficacious in removing lice. At these concentrations, brain AChE activity was marginally reduced, with reductions, from control values, of 25.6% and 14.6% for 1.0 and 2.0 mg/L respectively (Figure 5.31).

Control values for the two 1 hour propetamphos trials were combined (AChE μmoles/mL x min: 1 = 0.204 $\pm$ 0.048; 2 = 0.205 $\pm$ 0.044). At concentrations of 0.1 and 1.0 mg/L brain AChE levels were reduced from the control values by 13.2 and 16.7% respectively with no adverse effects noted in fish behaviour (Figure 5.32). At 3.0 mg/L, brain AChE activity was reduced by 39.7% from control values and fish were noted as being lethargic and showing signs of ataxia (loss of motor control) (Figure 5.32). However, after 1 hour, the fish began to recover when holding tanks were flushed with fresh seawater. Following the 30 minute exposures, brain AChE reductions were by 10.3% and 18.4% at 0.1 and 1.0 mg/L respectively with no adverse behaviour being noted (Figure 5.32).

Table 5.13. Mean Brain AChE activity (μmoles/mL x min) $\pm$ SD (n) in fish following 1 hour exposure to azamethiphos, CH65003 and Propetamphos (* = not tested).

Compound	Concentration Tested (mg/L)				
	Control	0.01	0.1	1.0	3.0
Azamethiphos	0.290 $\pm$ 0.083 (9)	0.270 $\pm$ 0.072 (10)	0.207 $\pm$ 0.043 (10)	*	*
Azamethiphos	0.115 $\pm$ 0.034 (5)	*	*	0.047 $\pm$ 0.015 (5)	0.047 $\pm$ 0.024 (5)
CH650003	0.182 $\pm$ 0.019 (5)	*	*	0.135 $\pm$ 0.034 (5)	0.155 $\pm$ 0.016 (5)[2]
Propetamphos	0.204 $\pm$ 0.046 (13)	*	0.177 $\pm$ 0.023 (5)	0.170 $\pm$ 0.036 (5)	0.123 $\pm$ 0.017 (5)
Propetamphos[1]	0.188 $\pm$ 0.021 (6)	*	0.168 $\pm$ 0.065 (6)	0.154 $\pm$ 0.066 (6)	*

[1] ½ Hour Exposures
[2] 2 mg/L

Table 5.14. Mean Brain AChE activity (μmoles/mL x min) $\pm$ SD in fish following 3 1 hour exposure to azamethiphos at weekly intervals (d = day, * = no exposure given).

Dose mg/L	No. Fish d1/d2/d3	Week 1	Week 2	Week 3
Control	10	0.173 $\pm$ 0.053	*	*
0.1	10/10/10	0.136 $\pm$ 0.028	0.118 $\pm$ 0.029	0.154 $\pm$ 0.030
0.3	10/10/10	0.099 $\pm$ 0.035	0.119 $\pm$ 0.040	0.106 $\pm$ 0.051
0.5	10/10/9	0.105 $\pm$ 0.045	0.033 $\pm$ 0.016	0.091 $\pm$ 0.020
1.0	10	*	0.051 $\pm$ 0.029	*
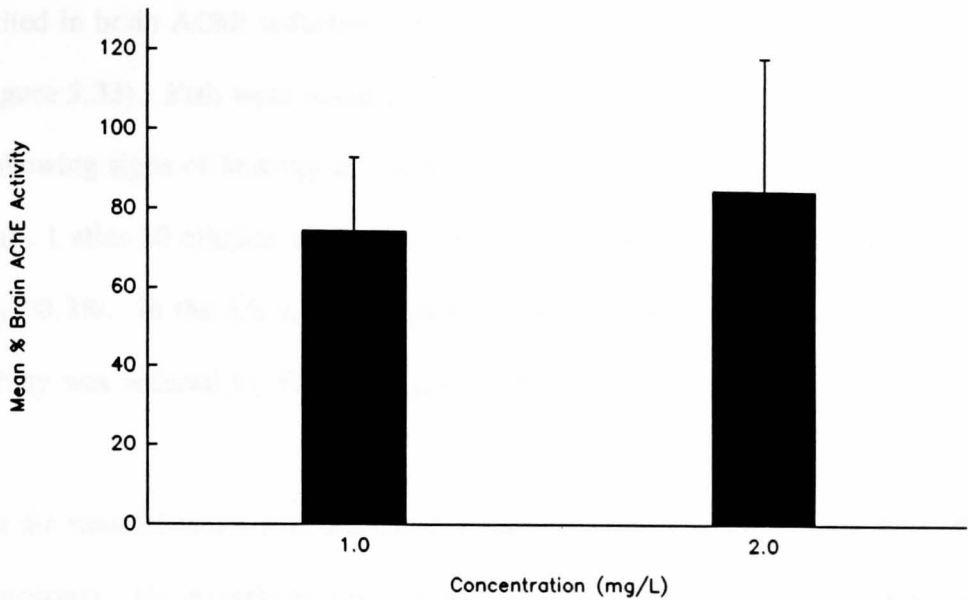
Figure 5.31. Mean % brain AChE activity (relative to control values) in salmon following exposure to C650003 (bars = SD).
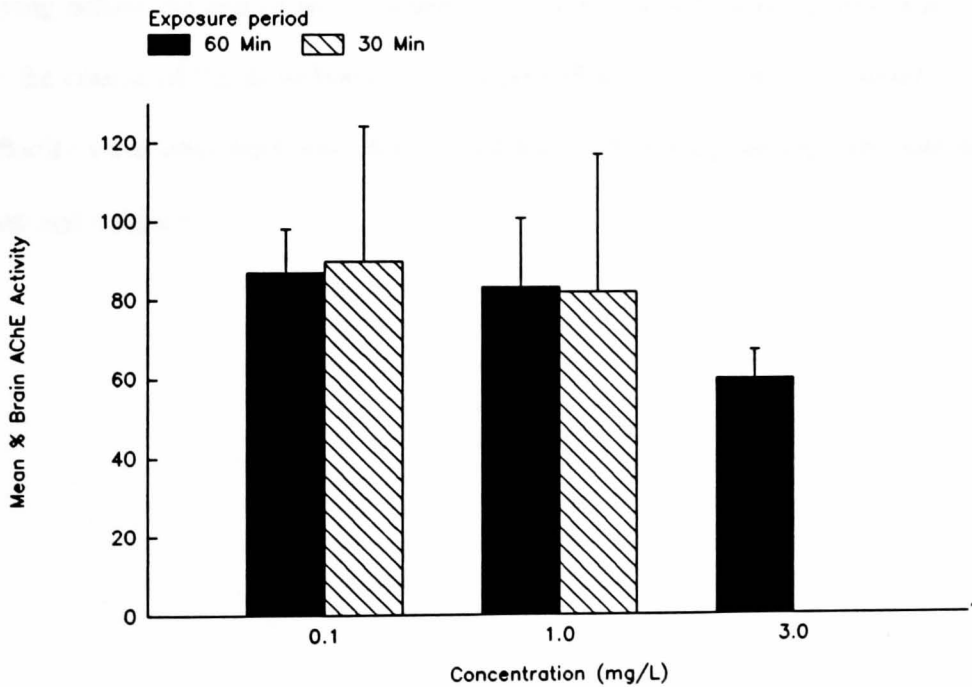

Figure 5.32. Mean % brain AChE activity (relative to controls) in salmon following exposure to propetamphos (bars = SD).

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Values obtained for the control brain AChE activity were somewhat more variable for the azamethiphos trials (single exposures) (Table 5.13). Concentrations of 0.01 and 0.1 mg/L resulted in brain AChE reductions of 6.4 and 28.6% respectively with no adverse effects (Figure 5.33). Fish were notably stressed in the 1.0 and 3.0 mg/L treatments with fish showing signs of lethargy and loss of coordination. Two fish died in the 1.0 mg/L group, 1 after 30 minutes and 1 after 50 minutes, with AChE activity being reduced by 60.3%. In the 3.0 mg/L treatment all fish died after 10 minutes, with brain AChE activity was reduced by 59.4% (Figure 5.33).

Results for the time tolerance trial are summarized in Tables 5.15 and Figure 5.33 (% AChE reductions). No mortalities were recorded during or after any of the treatments at 0.1 and 0.3 mg/L. At 0.5 mg/L mortalities were observed only after the second treatment; two fish before the end of the treatment, another fish within 24 hours and another after 48 hours. At 1.0 mg/L brain AChE activity was reduced by 70.3% with two fish dying before the end of the treatment. No appreciable loss in appetite was noted over the course of the experiment. At concentrations of 0.3 mg/L or higher signs of toxicity were consistent with those noted above, primarily lethargy and loss of coordination and balance.

5.3.3.2 Field Trials

Overall, brain AChE values

of substantial reductions. In

control, or post-exposure, values

data for all the treatments

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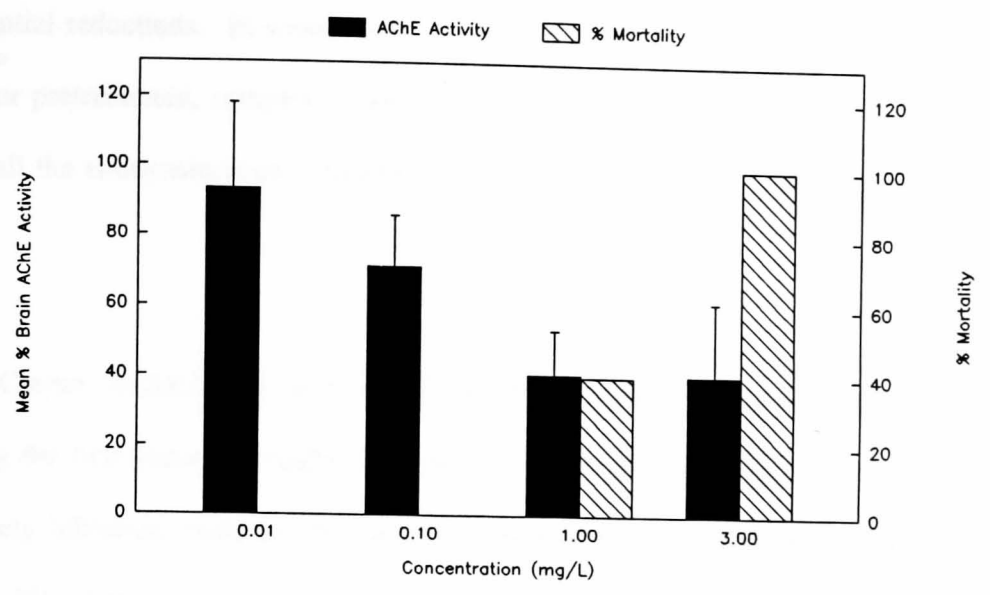
Figure 5.33. Mean % brain AChE activity (relative to control values) and % mortality in salmon following exposure to azamethiphos (bars = SD).
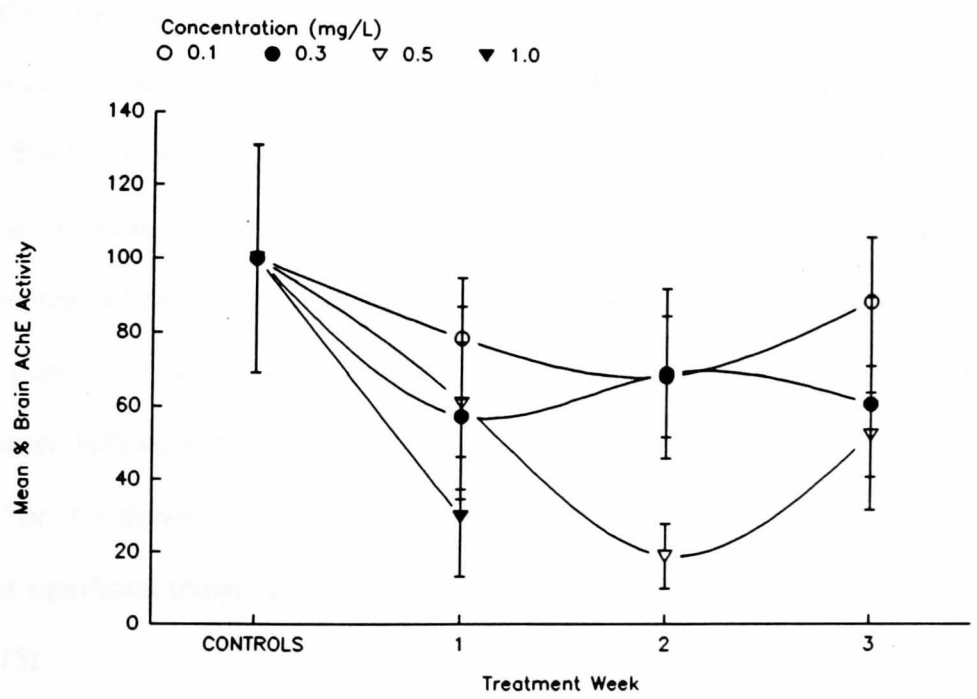
Figure 5.34. Mean % brain AChE activity in salmon following 3 weekly exposures to azamethiphos (bars = SD).

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5.3.3.2   Field Trials

Overall, brain AChE values appeared to remain near control values, with few instances of substantial reductions. In some instance, values were higher than that recorded for control, or pretreatment, samples. Table 5.15 summarizes the untransformed AChE data for all the sites/treatments. Percentage reductions are summarized in Figures 5.35 - 5.38.

At Loch Creran, AChE levels were not significantly altered from control values. Following the first treatment, slight reductions in AChE activity were observed immediately following treatment but had subsequently recovered within 24 hours (Figure 5.35). Immediately following the second treatment, AChE levels dropped by 33.4%, from controls, which was the greatest reduction recorded for the site. However, fish appeared to recover after 24 hours and were noted as feeding normally at the end of the treatment (Figure 5.35). Although a third treatment with azamethiphos was not carried out, a third treatment with DDVP was carried out by site staff. Before the end of this treatment (with DDVP), one of the treatments (cage 7) had to be aborted as the fish reacted negatively to the treatment (as mentioned previously). Despite the reaction by the fish, they appeared to recover as indicated by the relative reductions in brain AChE activity from samples taken 24 hours post treatment (listed as "3rd treatment" in Table 5.15). In addition, no fish mortalities were recorded following the aborted treatment. By comparison with control values obtained from the previous two treatments, reductions in AChE activity were not considered significant (range of -3.3% (therefore an increase) to 15.7% reduction) (Table 5.15).

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Results obtained for the Loch Duich trials are consistent with those obtained at Loch Creran. Following the first treatment, brain AChE levels appeared to decrease marginally but then recovered 24 hours following treatment (Figure 5.36). The greatest reductions were seen immediately after the second treatment where AChE levels had been reduced by 64.8%. However, fish recovered normally with AChE levels returning to pre-treatment levels 24 hours following treatment (Figure 5.36). A somewhat different trend was observed following the third treatment, with negligible reductions in AChE activity immediately after treatment followed by increased reductions with time. Reductions were greatest 1 week following treatment (23.1% reduction (from controls)) (Figure 5.36). It should be noted that "outgroup" samples varied somewhat from control values, with outgroup AChE values being higher than controls for the second and lower than controls for the third treatment. The use of the outgroup samples is questionable as fish from the site (and therefore of the same stock), could not be obtained which had not been previously exposed to DDVP.

Fish treated at Loch Seaforth, at 0.1 mg/L, showed little variation in AChE activity following treatment with azamethiphos (Figure 5.37). At 0.2 mg/L results were consistent with those obtained at Lochs Creran and Duich. Following the first treatment, at 0.2 mg/L, the greatest reduction was seen immediately following treatment (29.7 % reduction from controls). However, although recovery was observed, AChE activity did not completely return to pre-treatment levels (Figure 5.38). Following the second treatment, substantial reductions in AChE activity were observed 1 and 24 hours from treatment (≈ 34% from control levels). However, AChE activity subsequently returned to pre-treatment levels by the end of the 1 week recovery/observation period (Figure 3.38). Overall, reductions in AChE activity were

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consistently greater in fish treated at 0.2 mg/L than those treated at 0.1 mg/L.

In all treatments a hyperactive response of the fish was noted where fish jumped and moved along the surface of the cage water during treatment. This jumping behaviour was also observed during the 2nd, 3rd and, to a much lesser extent, 1st treatments at Loch Duich. Jumping behaviour was not observed during any of the Creran (azamethiphos) treatments. Where hyperactivity was observed, jumping commenced approximately 15-20 minutes from the onset of treatment and lasted approximately 30-40 minutes, decreasing in intensity with time. However, the duration and intensity was highly variable from treatment to treatment and site to site. As stated above, these responses were most evident during the Loch Seaforth treatments. Observations during these treatments indicated that higher concentrations of azamethiphos induced more intense hyperactivity (jumping). It was also thought that AQUAGARD® produced a more marked response.

Table 5.15. Mean brain AChE activity (μmoles/mL x min.) $\pm$ SD in fish following 1 hour exposure to azamethiphos at three sea cage grow out sites (* = no sample taken).

Site	Fish	1st Tmnt	2nd Tmnt	3rd Tmnt
Creran	Pre Treatment	0.185 ± 0.024	0.151 ± 0.029	*
	Post Treatment	0.164 ± 0.039	0.101 ± 0.029	*
	24 Hours	0.156 ± 0.028	0.145 ± 0.049	0.156 ± 0.028
	1 Week	0.195 ± 0.026	0.171 ± 0.018	*
	Out-Group	*	0.189 ± 0.039	*
Duich	Pre Treatment	0.176 ± 0.018	0.221 ± 0.039	0.212 ± 0.013
	Post Treatment	0.138 ± 0.024	0.078 ± 0.018	0.199 ± 0.030
	24 Hours	0.164 ± 0.031	0.181 ± 0.023	0.173 ± 0.037
	1 Week	0.200 ± 0.011	0.223 ± 0.026	0.163 ± 0.051
	Out-Group	*	0.204 ± 0.014	0.227 ± 0.026
Seaforth 0.1 mg/L	Pre Treatment	0.302 ± 0.038	0.271 ± 0.065	*
	Post Treatment	0.280 ± 0.028	0.278 ± 0.044	*
	24 Hours	0.323 ± 0.029	0.296 ± 0.027	*
	1 Week	0.308 ± 0.027	0.265 ± 0.048	*
	Out-Group	*	0.365 ± 0.028	*
Seaforth 0.2 mg/L	Pre Treatment	0.352 ± 0.025	0.340 ± 0.031	*
	Post Treatment	0.247 ± 0.040	0.233 ± 0.032	*
	24 Hours	0.262 ± 0.075	0.226 ± 0.052	*
	1 Week	0.278 ± 0.042	0.331 ± 0.030	*
	Out-Group	*	0.320 ± 0.039	*
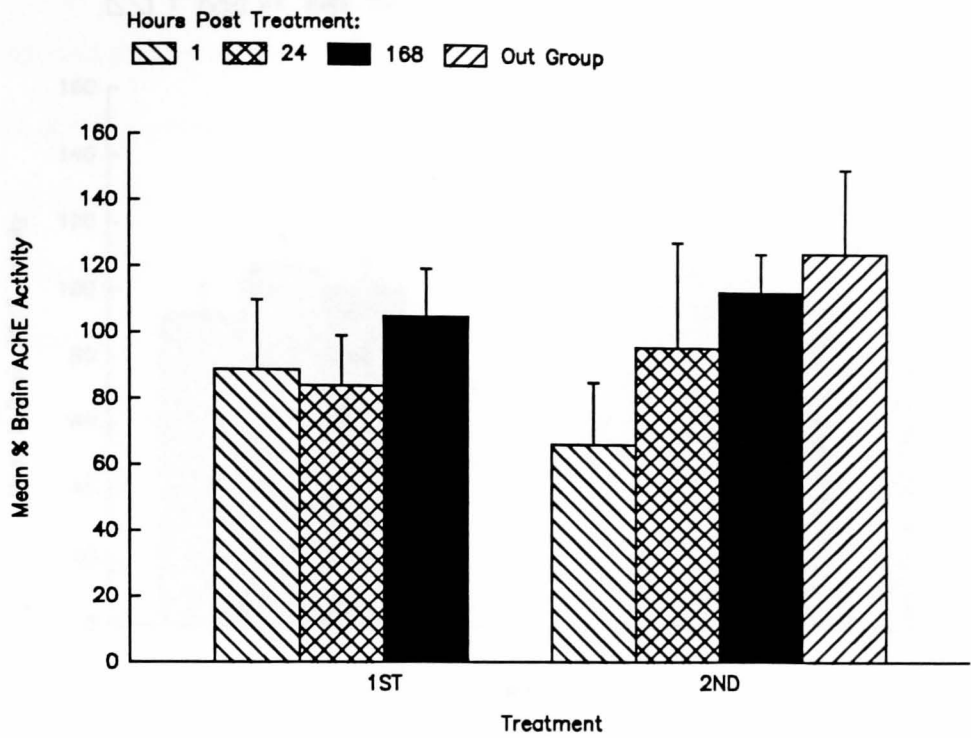
Figure 5.35. Mean % brain AChE activity (relative to control values) in salmon following treatment with azamethiphos (1-h @ 0.2 mg/L) at Loch Creran (bars = SD).
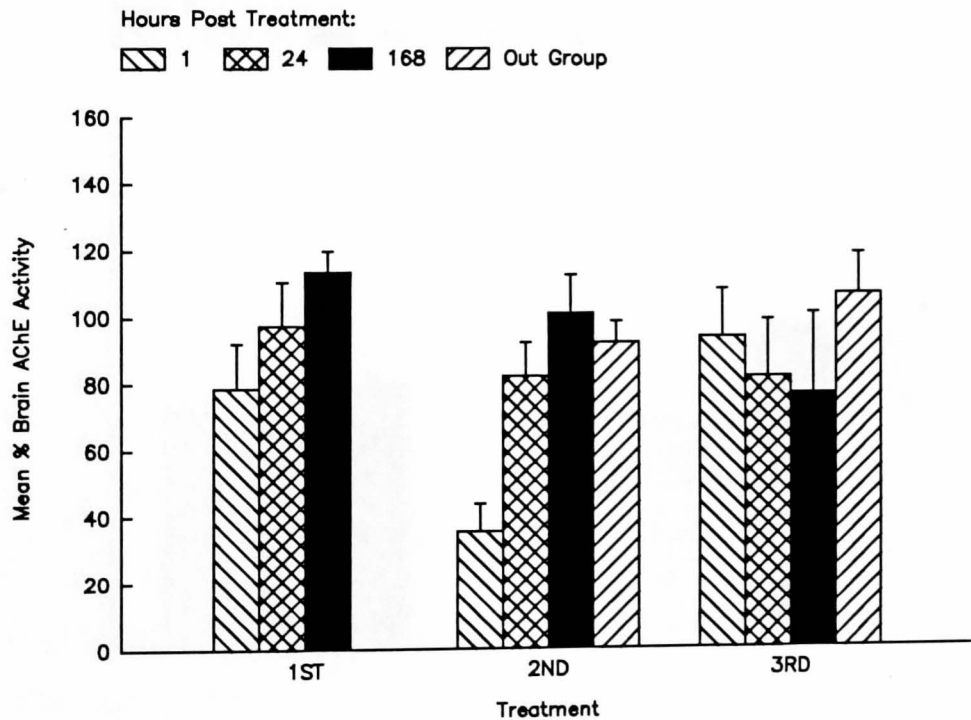

Figure 5.36. Mean % brain AChE activity (relative to controls) in salmon following treatment with azamethiphos (1-h @ 0.2 mg/L) at Loch Duich (bars = SD).
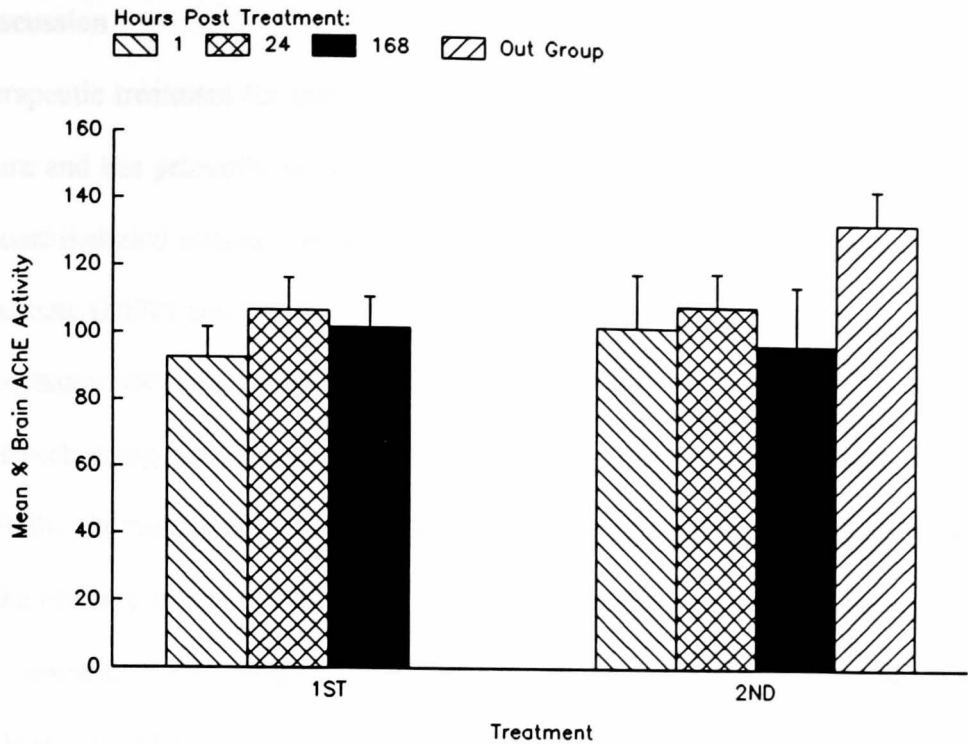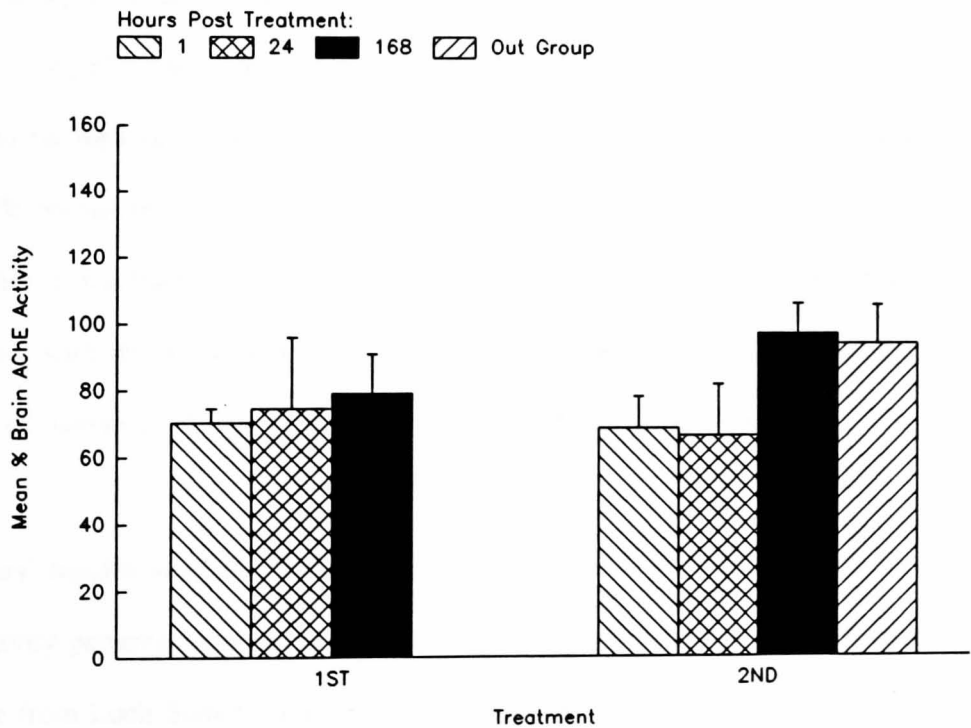
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Hours Post Treatment:
⬚ 1 ⬚ 24 ■ 168 ⬚ Out Group

Figure 5.37. Mean % brain AChE activity (relative to control values) in salmon following treatment with azamethiphos (1-h @ 0.1 mg/L) at Loch Seaforth (bars = SD).

Hours Post Treatment:
⬚ 1 ⬚ 24 ■ 168 ⬚ Out Group

Figure 5.38. Mean % brain AChE activity (relative to control values) in salmon following treatment with azamethiphos (1-h @ 0.2 mg/L) at Loch Seaforth (bars = SD).

5.4   Discussion

Chemotherapeutic treatment for parasitic disease has traditionally been associated with pond culture and has primarily been for the treatment of *Argulus* infestations. Early products used included organochlorine (OC) compounds such as dichloro-diphenyl-thrichloroethane (DDT) and benzene hexachloride (BHC) and, more recently, organophosphates (OP) (Kabata 1970). However, early workers quickly found that the efficacy of such compounds was reduced with the development of resistant populations (Kabata 1970). In the present study it would seem that the most significant modulating factor in the efficacy of novel chemotherapeutants was the development of resistance within the course of the investigation. Due to the sporadic and unpredictable availability of fish and/or lice and availability of test facilities, it was impractical to coordinate trials with respect to lice origin. Discussions with site managers and health monitors at all of the Loch Sunart sites revealed that standard DDVP treatments (1.0 mg/L - 1-h) were increasingly ineffective and that extended treatments (up to 3 hours) were necessary to significantly remove lice from infected fish. Given that the problem was widespread throughout the loch, it is assumed that lice from Loch Sunart, regardless of site, can be considered to represent a single population. Furthermore, it is questionable if lice from Lochs Creran and Aline represented significantly different populations, with respect to dichlorvos sensitivity, as indicated by reports by site managers of tolerance of lice (from both sites) to AQUAGARD[*] treatments.

Fishfarmers' reports were confirmed by Jones *et al.* (1992) who conducted a survey of lice sensitivity patterns collected from sites in several Scottish sea lochs. Of these lochs, lice from Loch Sunart were found to be the least sensitive (M. Jones pers. comm.). These findings were confirmed in the previous chapter and in the present

study. However, it is not known whether clinically oriented exposures (high dose, short exposure) would give the same resolution in delineating sensitivity patterns as would prolonged exposures as described by Jones *et al.* (1992) (low dose, long exposure). Despite such limitations, it is not surprising that a dose rate twice that recommended by Ciba-Geigy and reported by Rae (1979) was required to effectively treat infected salmon with DDVP. Although fish were found to tolerate a dose of 4.0 mg/L DDVP, at 8.0 mg/L half the experimental animals died. These findings are in agreement with Horsberg *et al.* (1987) who estimated the 1-h LC_{50} for salmon to be 8.7 mg/L. This would thus correspond to a, conservative, therapeutic ratio of two where resistant lice are to be treated. Given the error in calculating treatment volumes (Wells *et al.* 1990), the continued use of DDVP in such situations would be impractical.

The most severe implication of the development of resistance is side resistance between other organophosphates and the possibility of cross resistance to other classes of pesticides. Initially found to be highly efficacious, the therapeutic ratio of azamethiphos was found to be reduced from 30 to nearly 0 during the study period (3 years). Although toxic doses for either CH65003 and Propetamphos were not calculated, both compounds were found to be 100% efficacious at concentrations which were not toxic to fish and had little effect on brain AChE activity. Although further trials would be necessary to determine the acute toxicity of both compounds to fish, it is likely that resistance would inevitably reduce therapeutic margins. This is particularly true for propetamphos which was found to have a therapeutic margin which was similar to that determined for azamethiphos early on in the study.

As with azamethiphos, initial trials with resmethrin showed that the compound was

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highly effective, but over time higher concentrations were required to achieve optimum

efficacy (See Table 5.4). One possible explanation may be decreased activity, due to

degradation, of the resmethrin MS formulation between the two experiments (11

months). Alternatively, differences in the populations of lice at the time of collection

may reflect differences in susceptibility to the compound (Jones et al. 1992). Whether

or not the differences reported here are related to differential sensitivity between

populations is unknown. This latter hypothesis, although not to be ruled out, is

unlikely as the success of pyrethroids in the past has depended on high toxicity to pests

which show reduced sensitivity to OPs (Sawicki 1975; Carter 1989). Such

discrepancies stress the importance of quantifying toxicant concentrations following

bioassay oriented analysis.

The observed increase in toxicity of resmethrin with reduced temperatures is a common

phenomenon which has been reported for several pyrethroids. Mauck et al. (1976)

found that resmethrin was more toxic to several fish species at 12°C than 17°C. The

96-h LC_{50} for permethrin to rainbow trout is ten times lower at 5°C than 20°C

(Kumaraguru & Beamish 1981). The effect of temperature on the efficacy of

azamethiphos is unknown, but given that both DDVP and trichlorfon are more

efficacious at elevated temperatures (Horsberg & Høy, 1989) it is assumed that efficacy

of azamethiphos as well as other organophosphates, such as propetamphos and

CH65003, would also be temperature dependant.

Of the remaining pyrethroid compounds tested, therapeutic ratios were estimated to be

10 for lambda-cyhalothrin and 100 for PHRDL-C & D. Given the limitations in

estimating treatment volumes (as discussed earlier), compounds with such margins

would be better candidates for sea lice control and thus suggests that further testing under field conditions would be worthwhile. It is worth noting that such ratios would be considered representative, even in the unlikely event that cross resistance was present, since lice tested came from populations found to be resistant to DDVP and azamethiphos in the course of the present study. Furthermore, compounds such as cypermethrin (pyrethroid) are routinely used to control cattle ticks (*Boophilus microplus*) which are resistant to OP's (Worthing & Walker 1987). Thus, not only would such compounds show high efficacy in lice control, but would allow farmers to develop chemotherapeutic management strategies (in the absence of OP resistance) to delay the selection of resistance to any one given compound. The use of pyrethrum has been evaluated for clinical sea lice control in Norway and was found to be highly efficacious depending on the technique used (Boxaspen & Holm 1990; Anon. 1991a). However, the potential for the development of resistance to pyrethrins is great (Sawicki 1975) and if compounds, such as pyrethrum, were to be used for sea lice control, continual monitoring of lice sensitivity would be essential (see below). Although pyrethrum has been field tested in Norway, there are no reports on the presence of resistance to OPs to allow comment on cross resistance. The compound has been approved for field evaluation in the U.K. (F. Macdonald pers. comm.), thus data pertaining to performance under field condition, cross resistance, and the stability of sensitivity in lice populations is pending.

Similar to the results obtained in the previous chapter, the use of an emulsified formulation did not appeared to increase the toxicity of resmethrin to lice. However, it was noted that the MS formulation appeared to be more toxic to salmon than the EC formulation. Studies on the toxicity of pyrethroids to fish, following topical

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application, indicate that emulsifiers do not increase uptake, and therefore toxicity in fish (Bradbury, Coats & McKim 1986; Bradbury, Symonik, Coats & Atchison 1987). In light of this, the differences in the toxicity of the two formulations of resmethrin to fish in the present study are difficult to explain and may be related to changes in the stereochemistry of the molecule[8] in the new formulation (Bradbury *et al.* 1987). It might be hypothesised that the toxicity of resmethrin is more dependant on molecular chemistry than formulation, where the formulation is water soluble (Stammer 1959). It should be pointed out that the EC formulation was obtained 12 months after the MS formulation and the possibility of degradation can not be ruled out. However, similar results were obtained on the toxicity of the resmethrin MS formulation to salmon in subsequent trials (see chapter 7). In two separate exposures approximately 2 years apart (at similar exposure temperatures) salmon were found to tolerate 1 hour exposures at 1.0 mg/L and displayed similar signs of stress. These data would therefore suggest that the MS formulation was stable and that differential sensitivity in lice populations might be present and warrants further investigation, but also stresses the importance of internal standards.

In addition to the high efficacy observed with the pyrethroids, the milbemycin compounds SKB1 and SKB7 were also found to be highly efficacious, with therapeutic ratios of at least 10. The high efficacy of these compounds, combined with a different mode of action (and presumably different detoxification requirements) may represent a further class of compounds which might be considered for sea lice control. To date the only compound related to the milbemycins which has been evaluated for sea lice

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[8] The structure or stereochemistry of the active ingredient in either formulation was not disclosed by the manufacturer.

control is ivermectin (Palmer *et al.* 1987). However, there are no reports in the literature on the topical use of the avermectins or milbemycins for ectoparasite control in aquaculture. Conversely, the high activity of the milbemycins towards lice may also indicate the potential of the milbemycins as oral chemotherapeutants for sea lice control.

During the course of the time series trials, it was found that optimum efficacy was achieved 8 hours from treatment. A consistent observation in both trials was the residual effect in the SKB7 test groups, whereas a plateau in lice mortality was reached with both azamethiphos and resmethrin. One possible reason for the marginally higher efficacy of SKB7 1 week following treatment may be due to the stability of the molecule. Both DDVP and resmethrin are relatively unstable in sea water (Samuelsen 1987; WHO 1989a; Wells *et al.* 1990), whereas data on the structurally similar abamectin (a related avermectin compound) would suggest that SKB7 would be resistant to hydrolysis (Wislocki *et al.* 1989). Alternatively, SKB7 may be preferential toxic to the larval stages not included in lice counts. Organophosphates do not affect larval stages whereas avermectin-type pesticides do (Wootten *et al.* 1982; Palmer *et al.* 1987). It is possible that attached larval stages on fish treated with azamethiphos and resmethrin were unaffected by treatment giving higher lice counts at the end of the week, whereas SKB7 killed larval stages thereby resulting in 100% efficacy. Further studies were carried out specifically on the toxicity of these pesticides to larval stages of lice. These data form the basis of Chapter 7.

As mentioned above, all trials showed a delayed response with respect to lice toxicity following treatment. This presents a problem for the farmer who needs to monitor the

efficacy of treatment during the course of treatment. It is therefore suggested that fish are enumerated for lice at the end of treatment and the following day to estimate more accurately the effect from treatment.

As indicated by the laboratory trials, resistance also played a major role contributing to the efficacy of azamethiphos when tested at sea on-growing sites. Trials at Loch Seaforth clearly demonstrate that, where lice populations are sensitive to azamethiphos, it is highly efficacious. However, where lice show varying degrees of reduced sensitivity, as seen in the Loch Creran and Duich populations, efficacy of treatment will be dependant on the specific level of sensitivity and accuracy in estimating treatment dose.

Prior to the 1991 fallowing period, lice obtained from Loch Sunart were found to have significantly reduced sensitivity to DDVP (M. Jones pers. comm.). Fish health monitors at the sites indicated that overall lice numbers were much lower on fish in Loch Sunart farms following the fallow period. However, the number of lice present and the sensitivity of those present may not be correlated. The present findings suggest that although overall lice numbers were reduced, the sensitivity did not change appreciably. Thus, it is suggested that more stringent monitoring of lice sensitivity patterns will be required to gain needed information on the development of resistance at sites and to determine effective dose rates where chemotherapeutics are used. Such techniques of sensitivity testing should be developed alongside management control practices (fallowing, wrasse) to evaluate the effectiveness of such techniques on delaying the selection of resistance in lice populations.

Despite the reduced sensitivity of Creran or Duich lice, many of the treatments were highly effective, with post treatment reductions in lice in excess of 90%, suggesting that when treatment dose is correct, the compound is highly efficacious. However, in many instances efficacy of treatment at adjacent cages could be as low as 40%. It is difficult to speculate on the cause of such fluctuations, but identifying those factors which contribute to instances where treatment efficacy is reduced may aid in the development of more practical treatment protocols and therefore effective treatments.

Wells *et al.* (1990) showed that target concentrations of DDVP could vary by an order of magnitude during routine treatment practices. In the present study, efficacy of azamethiphos treatments at 0.2 mg/L ranged from 7.1 to 81.1% for Loch Creran and 41.9 to 96.6% at Loch Duich (24 hours from treatment). Such variation may be the result of variation in actual concentration from the target concentration, due to variation in the volume of water enclosed by tarpaulins once fitted. It is likely that the 0.2 mg/L concentration was close to the toxic threshold for lice. The best treatments occurred when the concentrations of azamethiphos approached or exceeded the target concentration. Where fitted tarpaulins enclosed larger than normal volumes of water, target concentrations would be reduced and therefore efficacy reduced. Such situations commonly occur where bags are fitted against tides, causing them to fill with unpredictably large quantities of water. Conversely, where tarpaulins are fitted on windy days or when seas are not calm, large air pockets are trapped in the tarpaulin, thereby reducing the volume of water contained. At present there is no method available to accurately estimate the volume of a cage prior to commencement of treatment, and farms must rely solely on the expertise and experience of site staff.

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It is difficult to know whether or not the volume estimates used were truly representative of the actual treatment volumes. Given that there were marginal differences between the sensitivity of the Creran and Duich lice, as determined *in vitro*, overall better efficacies at Loch Duich may suggest that volumes were underestimated at Loch Duich (smaller cages) and overestimated at Loch Creran (larger cages). The use of azamethiphos in future control regimens will be dependent not only on accurately assessing the sensitivity of local lice populations, but in accurately estimating treatment dose rates due to reduced therapeutic ratios.

Skirt treatments were found to be completely ineffective. This is not surprising given the uncertainty in estimating dose rates and the immediate dispersal of compound which would occur as a result of the open bottom of the skirt (Wells *et al.* 1990). Unless it is impossible to treat fish otherwise (as in the case of unusually strong currents) the use of skirts is not recommended.

Reinfection may have also contributed significantly to the effectiveness of azamethiphos treatments. In several instances, at Lochs Creran and Duich, lice numbers increased substantially within a week from treatment and in some cases exceeded pre-treatment levels. The Loch Seaforth treatments were probably enhanced by virtue of the fact that the entire site could be treated in two days. Large production sites such as Creran (32 pens) and Duich (60 + pens) may require 2 weeks for the entire site to be treated; leaving significant numbers of fish untreated undoubtedly maintains a reservoir of parasites which will quickly reinfect fish. Reinfection may also have occurred via untreated larval stages of lice which were unaffected by treatment.

The lack of effect on larval stages of lice following treatment with azamethiphos is not surprising given that DDVP does not appear to effectively kill these developmental stages (Wootten *et al.* 1982). Whether or not the mechanism responsible this phenomena is unique to organophosphates is unknown. Toxicity to larval stages is very significant where clinical treatments are concerned. If chalimus stages were killed during treatment, intervals between treatments would be longer thus reducing the number of overall treatments necessary. The present data supports the treatment protocol outlined by Wootten *et al.* (1982), suggesting that cages should only be treated when lice populations consist predominantly of adult and preadult lice to maximise efficacy.

Lastly it is unknown whether various environmental parameters such as temperature, pH, or salinity play a role in treatment efficacy. It is believed that, where lice are sensitive, potential reductions in efficacy as a result of such parameters would be less evident. However, where lice sensitivity is near to the safe allowable concentration (see below), effects from such environmental factors would be much more evident. Past work with both DDVP and trichlorfon has demonstrated that temperature has a direct effect on the efficacy of treatment (Horsberg *et al.* 1987). In the present study, it was impractical to study any temperature mediated effects due to the short time available. Efficacy of treatment with both trichlorfon and DDVP has been shown to be temperature dependent (Horsberg *et al.* 1987). These finding may lead one to conclude that temperature may also modulate the efficacy of azamethiphos. Thus, further work is warranted in this area.

The effect of oxygen content on treatment efficacy is unknown. Høy *et al.* (1991)

found that AChE inhibition in trout was significantly greater at low oxygen levels, but that recovery was the same (21 days) for fish exposed to DDVP at low (3.0 mg/L) and high (15.0 mg/L) oxygen levels. Given that DDVP has a high vapour pressure (1.6 Pascals (Pa) at 20°C (Worthing & Walker 1987), it might be speculated that vigorous oxygenation reduced the amount of available pesticide. Thus at low oxygen tensions, a greater concentration of pesticide would result in higher toxicity. Incidentally, azamethiphos has a much lower vapour pressure (0.0049 mPa (20°C), Worthing & Walker 1987), and combined with good water solubility characteristics would be much less susceptible to volatilisation. Alternatively, a more plausible explanation for increased toxicity at lower oxygen tension levels may be increased ventilation rate in the fish treated (Ott, Heisler & Ultsch 1980). However, decreases in ventilation rate due to low oxygen tension may also be negated by dose dependent decrease due to DDVP exposure (Verma & Tonk 1984). Regardless of the cause, recovery of AChE was independent of oxygen level, suggesting that AChE depression was due to the amount of pesticide taken up by the fish.

Although lice are known to be negatively affected by freshwater (McLean et al. 1990), site salinity is often unpredictable and long immersion times in fresh water would be required. Until further work is carried out on the synergistic effects of various pesticides and fresh water, the usefulness of information on fresh water immersion is of limited value. Lastly, factors such as pH and water hardness do not contribute to the toxicity of DDVP to salmonids (Johnson & Finley 1980) and therefore are not expected to contribute to treatment efficacy.

Although the acute toxicity of azamethiphos was not studied, laboratory trials suggest

that concentrations of 0.8 mg/L were found to result in significant mortalities. Treatments in excess of 0.2 mg/L, as used in the present study, are not recommended due to the uncertainty of estimating the treatment volume as discussed above. Given that such variation in estimating dose rate can occur, higher dose rates that 0.2 mg/L combined with large biomass contained in net pens and slow flushing following treatment may result in treatment mortalities.

Data on AChE activity in laboratory trials with azamethiphos suggests that fish were very tolerant of azamethiphos and were recovering within a week. Raverty (1987) found that when DDVP was used repeatedly at 3 and 6 day intervals for a period of 30 days, reductions in brain AChE were cumulative. No such cumulative effects were seen with azamethiphos. Where acute toxicity in fish treated with azamethiphos was observed, AChE levels were reduced by 60% from control values. These results are somewhat lower than that reported in the literature for salmonids where reductions of 75% have been correlated with acute toxicity (Coppage & Matthews 1974; 1975; Salte *et al.* 1987; Horsberg *et al.* 1989). However, although fish which show acute toxic effects from OP exposure display significant reductions in AChE, mortality and recovery are not necessarily related to AChE inhibition (Gibson, Ludke & Ferguson 1969; Coppage 1971). Other enzymological and physiological pathways disrupted by organophosphates include the gylcolytic sequence (Konar, 1969), serum transaminase (Verma, Rani & Dalela 1981a) liver, kidney and gill phosphatases (Verma, Rani & Dalela 1981b) serine proteases (Kraut 1977; Barrot & McDonald 1980) and lipid peroxidation in the central nervous system (Vadhva & Hasan 1986).

AChE depression in fish exposed to OPs is related to several factors which include

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dose, length of exposure, species and size of fish and oxygen tension (Weiss 1958;

Rath & Misra 1979: Verma *et al.* 1982; Høy *et al.* 1991). Recovery occurs by

regeneration of AChE, which is dependant on the same factors (Weiss 1958; Zinkl *et*

al. 1987). Raverty (1987) found that fish treated repeatedly at 3 and 6 day intervals

(1.0 mg/L DDVP) showed cumulative AChE depression, suggesting that fish may

become more susceptible to toxic AChE depression if they are not given sufficient

recovery periods. In the present study, fish were found to be more tolerant of repeated

exposure to azamethiphos and did not show cumulative AChE depression.

Most reports in the literature on the toxicity of OPs to fish and related sublethal effects

deal with tropical fish under conditions of chronic exposure. Such conditions are far

removed from the short intermittent exposures used for sea lice control and

physiological changes may not be significant. Most notable among these studies are the

findings of Pal and Konar (1985) who found that reductions in growth rate are related

to exposure. Vadhva & Hasan (1986) found that chronic exposure to DDVP of

Heteropneustes (Saccobranchus) fossilis resulted in dose dependant increases in lipid

peroxidation and decrease in phospholipids in regions of the central nervous system,

both thought to be primary indicators of irreversible brain cell damage which may also

be a contributing factor to DDVP toxicity.

Apart from the findings at Loch Seaforth, data from the various field treatments

suggest similar conclusions with respect to a lack of cumulative AChE depression.

Longer recovery periods required by fish from Loch Seaforth may have been related to

the small size of the fish, as size is inversely related to anti cholinesterase activity

(Rath & Misra 1979). However since the fish at Loch Seaforth had never been

exposed to OPs, unlike the fish at Lochs Creran and Duich which had been subjected to several exposures in preceding years, tolerance to organophosphate exposure was undeveloped or possibly due to their relatively smaller size. Factors contributing to the difference in AChE activity with respect to size include large gill surface area relative to body size, higher rate of metabolism and smaller lipid pool in the body (Horsberg *et al.* 1989).

In the present study, predominant signs of toxicity from azamethiphos included lethargy and loss of orientation and equilibrium. These findings are consistent with general behaviour in fish succumbing to organophosphate toxicity (Symons 1973; Pal & Konar 1985; Raverty 1987). Interestingly, fish treated with OPs in laboratory trials did not show signs of hyperactivity or "jumping" as seen in the cage trials. Raverty (1987) noted that fish exposed to DDVP (laboratory conditions) showed similar signs of hyperactivity but not leaping from the water. It is possible that the absence of this behaviour is artifactual and related to modifications in behaviour related to the confines of laboratory tanks, as DDVP treatments carried out on farm sites are notably stressful to fishes (Bjordal, Fernø, Furevik & Huse 1988). Differences seen in the responses of Loch Seaforth fish and fish treated at Lochs Creran and Duich may have been due to variation in fitting tarpaulins (Wells *et al.* 1980) or differences in the size of fish at each of the sites (Rath & Misra, 1979). However, it is not known if past exposure predisposes fish to react either positively or negatively to OP toxicity.

6 The Efficacy of Alternative Chemotherapeutants for the Treatment of Sea Lice Infections of Atlantic Salmon: Oral Treatments

6.1 Introduction

In the preceding review on chemotherapeutic control of sea lice (Chapter 3), the use of tarpaulin style, or "bath treatments" was shown to have several disadvantages. Although many such disadvantages, such as low therapeutic ratios and lack of toxic effects to all stages of lice, relate specifically to compounds such as DDVP, others are concerned specifically with the method of application and include difficulties in handling tarpaulins, the requirement for several days (possibly weeks) to treat an entire site and difficulties in estimating correct dose rate. Given these limitations, an oral chemotherapeutant has been sought by fish farmers for many years.

The systemic application of neurotoxins in order to effect clinical control of ectoparasites presents many problems, most of which are associated with the non-selective toxicity of these compounds to both parasites and hosts. The first oral chemotherapeutant evaluated for sea lice control was trichlorfon (Brandal & Egidius 1977). However, the compound was found to be unsuitable due to toxic side effects in treated fish, primarily blindness. Recently, the compound diflubenzuron (DFB) has also been evaluated experimentally as an anti-lice oral chemotherapeutant (Høy & Horsberg 1991). Since DFB inhibits chitin synthesis it is selective to arthropods and relatively non toxic to salmon (96-h LC_{50} 140 mg/L; Worthing & Walker 1987). However, the compound was found to be poorly absorbed by salmon and required relatively high dose rates (14.0 mg/kg for 14 consecutive days) with a large portion of

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the administered dose passing through the gut. Consequently, the potential for the compound to accumulate below salmon pens, leading to toxicity to marine crustaceans questions its use in salmon farming (Roth *et al.* in press).

Ivermectin, representative of the most recently developed avermectin class of pesticides, has also been experimentally evaluated as an oral sea lice chemotherapeutant (Palmer *et al.* 1987; Smith *et al.* in press) and is now widely used for sea lice control in Ireland (Siggins 1990; Clover 1991; O'Sullivan 1991). One primary concern regarding the use of ivermectin for sea lice control is the potential for the compound to accumulate in marine sediments, as observed in terrestrial model systems (Halley *et al.* 1989a; Roth *et al.* in press). Furthermore, it should also be noted that under current treatment regimens, the compound is used at relatively low dose rates extended over long periods of time (up to 10 months in some instances) (Smith *et al.* in press). Although the compound is highly efficacious in removing lice, such treatment practices are extremely conducive to the development of resistance in pest populations (Roush & Daly 1990; Tabashnik 1990).

Given these limitations, it was hypothesized that a more pragmatic approach would be to develop an oral chemotherapeutant, or treatment regimen, which could be used on a single treatment basis. Thus a series of experiments were designed to assess the toxicity/efficacy of ivermectin and two compounds, SKB5 and SKB8, from the structurally similar milbemycin group (Fisher, 1985), when administered as a single application spread over three days. In addition, two pyrethroid compounds were chosen, PHRDL-C and PHRDL-D, to be tested in a similar fashion as they showed the widest therapeutic ratio of the compounds screened during the course of the project.

6.2 Methods

6.2.1 General

Initially a series of trials was carried out using simulated in-feed models in the laboratory. This approach was taken for several reasons. First of all, there are obvious restrictions on testing unlicensed compounds at farm sites. Secondly, experience has shown that fish which have been acclimated at sea for several months are reluctant to accept feed once transferred to laboratory tanks for a considerable period of time (several months). Furthermore, restrictions on the movement of salmon stock from third party sources (to prevent disease transmission between sites) severely limited size/year class of fish available for in feed efficacy studies.

Initially two laboratory model systems were evaluated, which were designed to overcome problems associated with introducing oral medication to laboratory fish populations. Both models involved administering drug to fish under anaesthetic, the first via an oral gavage (stomach tube), the second by placing capsules containing the appropriate drug into the oesophagus. Where efficacy was to be evaluated, fish were infected with sea lice (as described previously) one day prior to drug administration.

The advantages of both techniques were that fish could be handled to allow accurate weight measurement (therefore allowing accurate dosing) and lice counting (to evaluate efficacy). Unfortunately many problems were encountered with both systems, primarily due to regurgitation of stomach contents, a common problem where fish are force fed (Taraschewski et al. 1988). Initial experiments using the gavage technique showed that fish were regurgitating most, if not all, of the compound administered, which was affecting lice topically in a dose dependant fashion at concentrations similar

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to those found to be efficacious in one hour bath treatments. Administration of medication via a capsule did not confer any advantage as a 30%+ regurgitation rate was observed which made it impossible to assess any clinical effect.

Following the attempts at simulating in-feed treatments, fish were then made available which had recently smolted (at a hatchery) and were successfully acclimated (i.e. showed a normal feeding response) at the Sea Life Centre at Oban. The above trials were then repeated by incorporating medication into the feed and are described below.

All of the trials were based on laboratory infections. Atlantic salmon (*Salmo salar*) smolts were obtained from hatchery reared stock and unless stated otherwise were transferred to holding tanks at the Sea Life Centre. For each of the trials, fish were acclimated for several weeks during which they were offered pelleted feed, *ad libitum*, until a regular, consistent, feeding behaviour had been established.

Once acclimated, fish were randomly divided into groups of 13 and infected with adult and preadult sea lice (*Lepeophtheirus salmonis*). Lice used for the infections were collected from salmon grow-out sites on the west coast of Scotland as described previously. To infect fish, the water volumes in the holding tanks were temporarily adjusted to 200 L (from 540 L) and between 225 - 325 lice were added to each of the tanks (or 17 - 25 lice/fish, numbers given below). The tanks were then allowed to stand static, with aeration, for four hours, after which water flows to the tanks were reinstated.

Twenty four hours following infection (sample day "1"), three fish from each of the

groups were removed (selected at random), killed with a blow to the head, weighed and the number of lice counted. The weights obtained were then used for dose calculations in preparing medicated diets. The remaining fish in each group (10) were then offered medicated diets (compounds and dose rates given below) for three consecutive days. For all experiments, fish were fed with medicated feed at a feeding rate of 1.5% body weight per day (% bdy wt/d). Pellets were medicated by incorporating compounds into a pre-cooled (<35 °C) 5.0% gelatin solution, which was added to the feed (incorporation rates given below) and gently mixed. Pre-weighed feed lots were maintained in sealed glass containers, refrigerated (and kept in the dark) until used. During treatments, observations were made of the behaviour of the fish and in particular any changes in feeding patterns.

Following the treatment period (i.e. on day 4), fish were returned to their normal diets. Unless stated otherwise, lice counts were made 4, 7 and 14 days following the last feeding (sample days 7, 10 & 17 from the beginning of the experiment). Lice were counted by removing fish from tanks with a hand net and anaesthetizing them in a mild benzocaine (0.01%) solution, counting lice by careful visual observation and returning the fish to their respective tanks for recovery. On the last sampling for all experiments fish were killed with a blow to the head, weighed, and lice counted.

6.2.2 Compounds Tested

6.2.2.1   PHRDL-C & PHRDL-D

6.2.2.1.1   Experiment 1

For experiment 1, only PHRDL-D (5.0% w/w a.i. fine powder, obtained from Peter Hand Animal Health) was evaluated. Fish were infected at a rate of 17 lice/fish.

Medicated pellets were coated with gelatin at a rate of 1.0 mL per 12.5 g of feed. Dose rates studied were 0.0 (control), 0.025, 0.05 and 1.0 mg/kg body weight/day (mg/kg bdy wt/d). Fish were sampled for lice 4, 7 and 14 days from the last medicated feeding.

6.2.2.1.2   Experiment 2

For experiment 2 only PHRDL-D (5.0% w/w a.i. fine powder, obtained from Peter Hand Animal Health) was evaluated. Fish were infected with lice at a rate of 25 lice/fish. Medicated pellets were coated with gelatin at a rate of 1.0 mL per 12.5 g of feed. Dose rates studied were 0.0 (control), 0.1, 0.5 & 1.0 mg/kg bdy wt/d. Fish were sampled for lice 4, 7 and 14 days from the last medicated feeding.

6.2.2.1.3   Experiment 3

For experiment 3 both PHRDL-C (10 % w/w a.i. fine powder) and PHRDL-D (100 % technical compound) were evaluated. Both compounds were obtained from Peter Hand Animal Health. Trials were carried out at the Machrihanish Field Station on the west coast of Scotland. The tanks at Machrihanish are square 1.0 m^2 fibre glass tanks with a depth of 0.3 m (300 L). All tanks were supplied with pumped flow through natural sea water. Fish used for the trial had been acclimated at the facility for several months.

The volume of the tanks was reduced to 200 L and the fish infected at a rate of 19 lice/fish. There were 4 groups in total: 0.0 (control); 1.0 mg/kg bdy wt/d PHRDL-D (gelatin coating); 1.0 mg/kg bdy wt/d PHRDL-C (gelatin coated) and 1.0 mg/kg bdy wt/d PHRDL-C (oil coated). Medicated pellets were prepared by Peter Hand Animal

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Health as follows: medicated pellets were coated with gelatin at a rate of 1 mL per 10 g of feed. Pellets were coated with medicated oil (food grade vegetable oil) at a rate of 5% (w/w). Fish were fed medicated feed at a feeding rate of 1.0 % bdy wt/d for three consecutive days. All medicated feed lots were packed in glass containers as described above. Fish were sampled for lice on day 1 (of medication) and then 1 and 4 days from the last medicated feeding (sample days 4 & 7 from the beginning of the experiment).

6.2.2.2 Ivermectin

One trial was completed with ivermectin. The experiment was run alongside experiment 2 (above) and the same protocols were used (including the same reference control group). Ivermectin was obtained as a 1.0 % a.i. w/v injectable solution (Merck Sharp and Dohme Ltd.). The appropriate amount of compound was mixed into a pre-cooled gelatin (5%) solution which was coated onto pre-weighed feed lots (1.0 mL gel per ivermectin mixture per 12.5 g of feed). Dose rates studied were: 0.05, 0.1 & 0.5 mg/kg bdy wt/d. Fish were sampled on day 1 (of medicated feeding) and 4, 7 & 14 days from the last medicated feeding (sample days 7, 10 & 17 from the beginning of the experiment).

6.2.2.3 SKB5 & SKB8

SKB5 (100% a.i. technical compound) and SKB8 (20% a.i. w/w fine powder) were obtained from SmithKline Beecham Pharmaceuticals. Both compounds were solubilized in ethyl alcohol (ETOH) and subsequently diluted by adding the appropriate volume of stock to a 5% pre-cooled gelatin solution. The gelatin solution was then coated (1.0 mL per 12.5 g of feed) onto pre-weighed lots of pellets and gently mixed.

Unmedicated ETOH was added to the gelatin used to coat pellets for the control group. Dose rates for both compounds were (active) 0.0 (control), 0.01, 0.1 and 1.0 mg/kg bdy wt/d. Fish were fed the medicated diets at a feeding rate of 1.5 % bdy wt/d.

Fish were sampled for lice on day 1 (of medication) and 1, 4, 7, 14 and 28 days from the last medicated feeding (sample days 4, 7, 10, 17, 24 from the beginning of the experiment).

6.2.3 Data Analysis

Analysis of variance (ANOVA) was used to compare lice response in various groupings of data sets. Multiple comparisons were performed using the Tukey HSD (honestly significant difference) multiple comparison procedure. Due to small numbers of lice encountered on many fish, and a high degree of heteroscedasticity (i.e lack of homogeneity in sample variances), data were transformed using the Freeman and Tukey (1950 - cited in Zar 1990) modification of the square root transformation as follows:

$$X' = \sqrt{X} + \sqrt{(X + 1)}$$

Homogeneity of variance was confirmed using the F_{max} test (Sokal & Rohlf 1987) (variance ratio test) for $P < 0.05$. Randomness in the distributions was compared using the Chi square test (variance to mean ratio), calculated using the following equation:

$$x^2 = s^2/X \times v$$

where

x^2 = test statistic

s^2 = variance

X = mean

v = degrees of freedom (n-1)

Significance for the test statistic for clumped, random or regular distributions was calculated for $P < 0.05$[9]. Standard students t-tests were used for pair-wise comparisons of transformed data.

Mean efficacy (% reduction in lice) was calculated as described previously, using day 1 lice counts for individual groups to estimate % reductions. Reductions were then corrected for control mortality, estimated (% lice reduction) from the control group proper, by subtracting the calculated efficacy in the control group from the calculated efficacy in the test group.

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[9] This equation was taken from Fowler, J. & Cohen, L. (undated) Statistics for Ornithologists British Trust for Ornithology, Guide No. 22.

6.3 Results

6.3.1 General

Temperatures, salinities and fish weights recorded during all of the experiments are given in Table 6.1. Temperatures throughout the various trials ranged from 9.5 to 14.1°C. Salinity ranged from 31.0 to 35.1‰. Fish weight was somewhat more variable and ranged from 82.2 to 173.4 g (Table 6.1).

Table 6.1. Temperature, salinity and mean fish weights recorded during sea lice oral chemotherapy trials.

Compound/ Trial	Temperature °C ± SD	Salinity ‰ ± SD	X Fish Weight g ± SD
PHRDL-C & D			
Exp. I	12.0 ± 0.5	31.0 ± 1.0	157.3 ± 29.3
Exp. II	9.5 ± 1.5	32.5 ± 2.5	173.4 ± 43.7
Exp. III	14.1 ± 0.2	35.1 ± 0.3	100.0 ± 15.6
Ivermectin	9.5 ± 1.5	32.5 ± 2.5	156.8 ± 32.8
SKB5 & 7	9.5 ± 1.0	32.5 ± 1.5	82.2 ± 17.7

6.3.2 Experimental Populations

There was a high degree of variation in the success of infections with infection rates of 17, 27, 25 & 25 (lice/fish) resulting in mean parasite burdens of 13.4, 27.7, 8.6 and 14.5 (lice/fish) respectively (Table 6.2). In three of the 4 infections, lice were significantly overdispersed (clumped). A multiple range test of the transformed data (Tukey, $P < 0.05$) showed that the second infection (27 lice/fish) resulted in significantly higher lice burdens on fish than either infection at 25 lice/fish. Infection 3 (at the Sea Life Centre) resulted in significantly lower parasite burdens than infection 4 (at Machrihanish) (transformed data, $t = -2.59$, $P = 0.02$). Parasite burdens were not significantly different between the 17 lice/fish infection and either of the 25 lice/fish infections (Tukey, $P < 0.05$).

A significant reduction in lice numbers was observed in all of the control groups over time (Table 5.3). In most instances lice numbers were significantly lower at sampling day 10 (Tukey, $P < 0.05$). Control mortality rates were highest during the SKB5 & 8 trials in which lice numbers were significantly lower on sampling day 4 (Tukey, $P < 0.05$).

Table 6.2. Infection results of laboratory infections with wild stock sea lice and sea water acclimated smolts. * = significantly clumped or overdispersed (Chi Squared, $P < 0.05$).

	Number of Lice/Fish			
	Infection 1	Infection 2	Infection 3	Infection 4
Infection Rate	17	27	25	25
Post infection				
X (SD)	13.42 (4.60)	27.67 (15.32)	8.62 (3.76)	14.5 (9.17)
n	12	21	21	12
Median	12.5	26.0	9.0	11.0
Min - Max	6-21	8-67	3-18	6-37
s^2/X x v	17.4	169.6*	32.8*	63.8*

X = mean, s^2 = variance, v degrees of freedom (n-1), n = sample size

Table 6.3. Mean number of lice/fish (SD) in each of the control sampling groups for each of the feed trials. Standard deviations are given in parenthesis. * = significantly different from day 1 counts (Tukey, $P < 0.05$).

Compound/ Trial	Sample Day (from beginning of Exp.)					
	1	4	7	10	17	24
PHRDL-D/C						
Exp. 1	10.3 (2.9)	-	9.0 (2.4)	7.3 (2.3)*	5.2 (2.2)*	-
Exp. 2 + Ivermectin	20.3 (10.0)	-	15.5 (4.1)	10.2 (4.1)*	7.4 (3.8)*	-
Exp. 3	9.0 (2.7)	9.1 (3.9)	5.6 (3.0)	-	-	-
SKB5/8						
Exp. 1	8.3 (8.4)	2.7 (2.2)*	1.5 (1.3)*	0.9 (1.1)*	0.9 (0.6)*	1.1 (1.4)*

6.3.3 Individual Treatments

Lice counts recorded for all of the experiments are summarized in Tables 6.4 (pyrethroids) and Table 6.5 (avermectin/milbemycins). Results were only interpreted for data points in which control lice counts are not significantly reduced from the day 1 counts (as discussed above, see Table 6.3), and are summarized for individual experiments below.

6.3.3.1   PHRDL-C & PHRDL-D

Results from experiments 1 and 2 (PHRDL-D) are summarized in Figures 6.1a and 6.1b. However, due to the effects of time on the overall numbers of lice in the control groups, only data for sample days 1 and 7 were used for statistical comparisons between the treatment groups (concentrations) which are summarized in Figures 6.2a and 6.2.b.

Overall, lice numbers on day 1, in both trials, did not differ significantly between the treatment groups (Exp. 1, F = 0.706, P = 0.575; Exp. 2, F = 0.388, P = 0.765). However, a consistent dose response was observed in the treatment groups on day 7 with respect to dose (see Figures 6.2a & b; Table 6.6). For experiment 1, results from the multiple range test (Tukey, P < 0.05) showed that lice numbers were significantly reduced from the controls in the 0.1 mg/kg bdy wt/d group and that for experiment 2, lice numbers were significantly lower in the 0.5 and 1.0 mg/kg bdy wt/d groups. Throughout both trials, all fish appeared unaffected by the treatment and showed no signs of abnormal behaviour.

Table 6.4. Mean number of lice/fish (SD) following oral treatment with the pyrethroid compounds PHRDL-D (Exp. 1, 2 & 3) and PHRDL-C (Exp.3). NS = no sample taken

Dose Rate (mg/kg bdy wt/d)	Sample Day (from the beginning of the experiment)				
	0	1	4	7	14
Experiment 1 (PHRDL-D)					
0.0	10.3 (2.9)	NS	9.0 (2.4)	7.3 (2.3)	5.0 (2.2)
0.025	15.0 (3.0)	NS	6.3 (3.2)	5.4 (2.6)	3.1 (2.2
0.05	15.3 (5.1)	NS	6.4 (2.4)	5.7 (1.8)	3.8 (1.2)
0.1	13.0 (7.0)	NS	4.3 (1.8)	4.3 (2.5)	2.6 (1.6)
Experiment 2 (PHRDL-D)					
0.0	20.3 (10.0)	NS	15.5 (4.1)	10.2 (4.1)	7.4 (3.8)
0.1	27.7 (4.2)	NS	12.9 (4.7)	11.7 (4.0)	8.1 (3.8)
0.5	34.0 (16.1)	NS	8.5 (2.0)	6.1 (2.7)	5.4 (2.9)
1.0	29.3 (23.2)	NS	2.3 (2.1)	1.6 (1.8)	1.0 (1.6)
Experiment 3 (PHRDL-C/D)					
0.0	9.0 (2.7)	9.1 (3.9)	5.6 (3.0)	NS	NS
D-GEL	23.7 (13.5)	11.7 (5.6)	11.5 (5.6)	NS	NS
C-GEL	7.3 (1.5)	13.9 (4.8)	11.5 (6.0)	NS	NS
C-OIL	18.0 (2.0)	13.5 (2.3)	8.2 (4.2)	NS	NS

Table 6.5. Mean number of lice/fish (SD) following oral treatment with ivermectin, SKB5 and SKB8. NS = not sampled.

Dose Rate (mg/kg bdy wt/d)	Sample Day (from the beginning of the experiment)					
	0	1	4	7	14	28
Ivermectin						
0.0	20.3 (10.0)	NS	15.5 (4.1)	10.2 (4.1)	7.4 (3.8)	NS
0.05	22.0 (13.8)	NS	26.4 (9.2)	23.0 (8.6)	16.0 (7.2)	NS
0.10	15.3 (3.1)	NS	16.7 (5.8)	14.0 (4.1)	7.0 (3.7)	NS
0.50	45.0 (19.1)	NS	31.3 (8.3)	23.7 (5.1)	13.5 (0.7)	NS
SKB5						
0.0	8.3 (8.4)	2.7 (2.2)	1.5 (1.3)	0.9 (01.1)	0.9 (0.6)	1.1 (1.4)
0.01	8.0 (1.7)	6.8 (3.1)	4.5 (2.8)	1.6 (1.7)	0.9 (1.2)	0.4 (0.5)
0.1	5.3 (3.2)	3.3 (2.3)	2.7 (1.5)	2.2 (1.2)	1.5 (1.0)	0.2 (0.4)
1.0	8.3 (2.1)	6.3 (2.1)	3.3 (0.6)	2.3 (0.6)	1.7 (1.5)	1.3 (0.6)
SKB8						
0.0	8.3 (8.4)	2.7 (2.2)	1.5 (1.3)	0.9 (1.1)	0.9 (0.6)	1.1 (1.4)
0.01	10.3 (0.6)	2.8 (1.8)	2.1 (2.0)	1.1 (1.0)	1.1 (0.6)	0.8 (1.0)
0.1	9.3 (4.7)	3.8 (1.3)	2.1 (1.7)	1.6 (1.3)	0.7 (0.7)	0.2 (0.4)
1.0	10.7 (1.5)	8.5 (7.8)	6.0 (7.0)	5.5 (5.0)	1.0 (0.0)	0.5 (0.7)
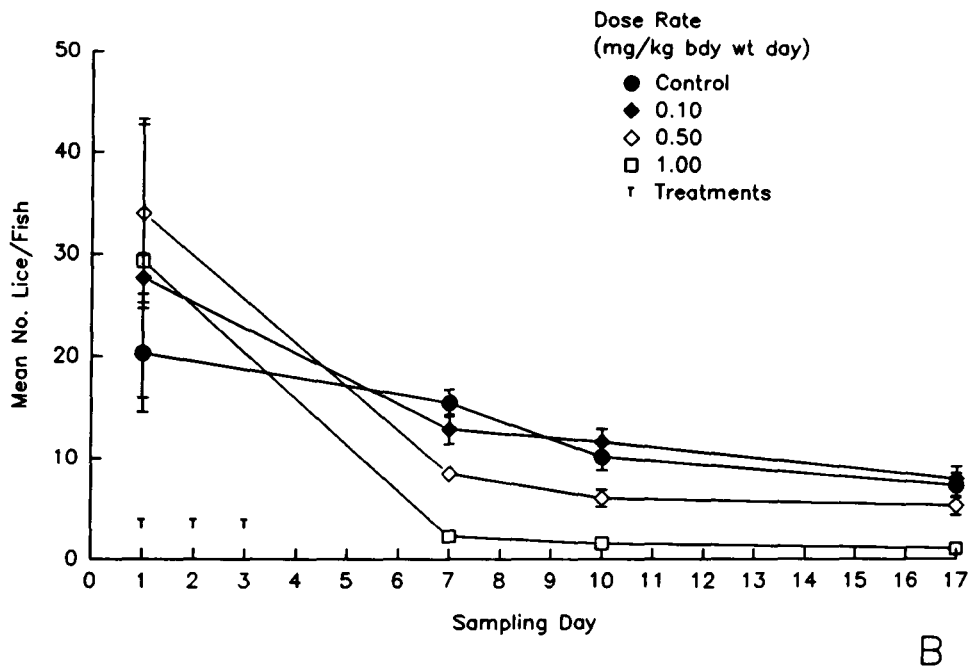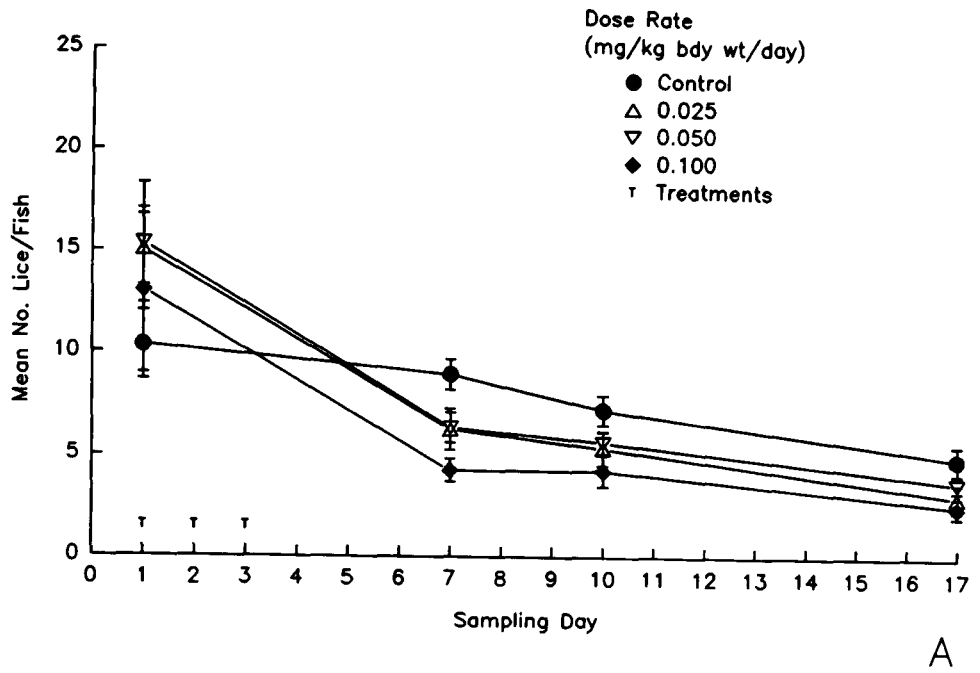
Figure 6.1. Mean reduction in sea lice infecting Atlantic salmon following oral treatment with PHRDL-D. A, experiment 1; B, experiment 2 (bars = SD).

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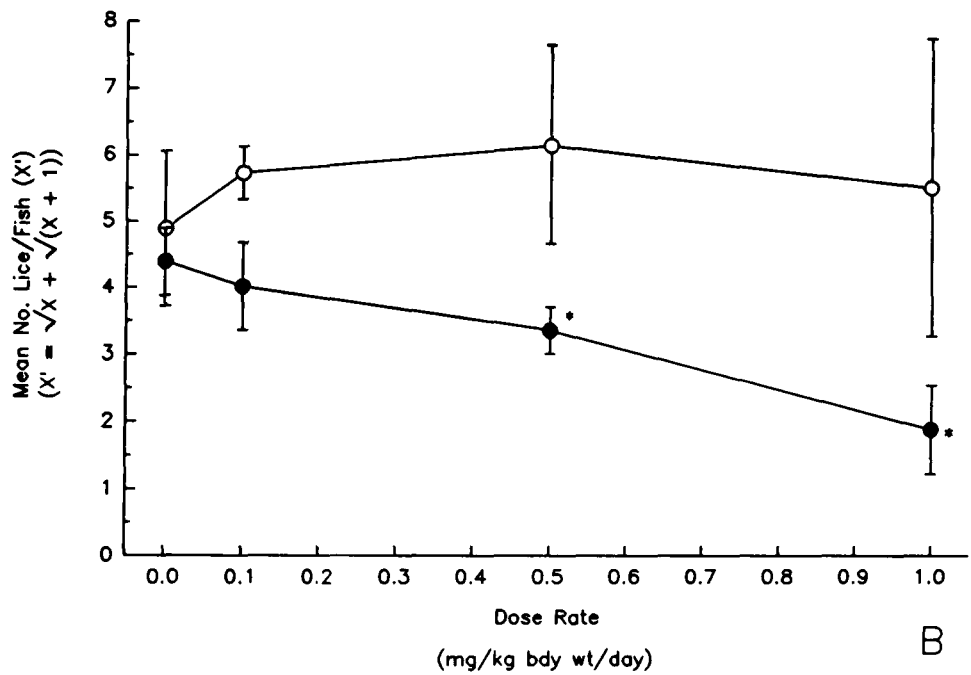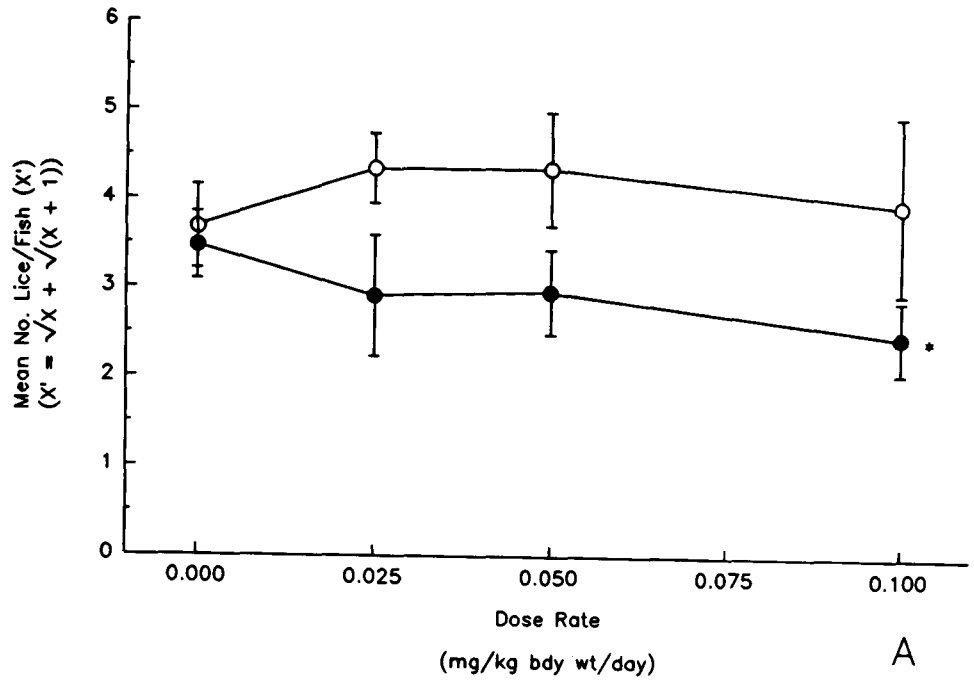
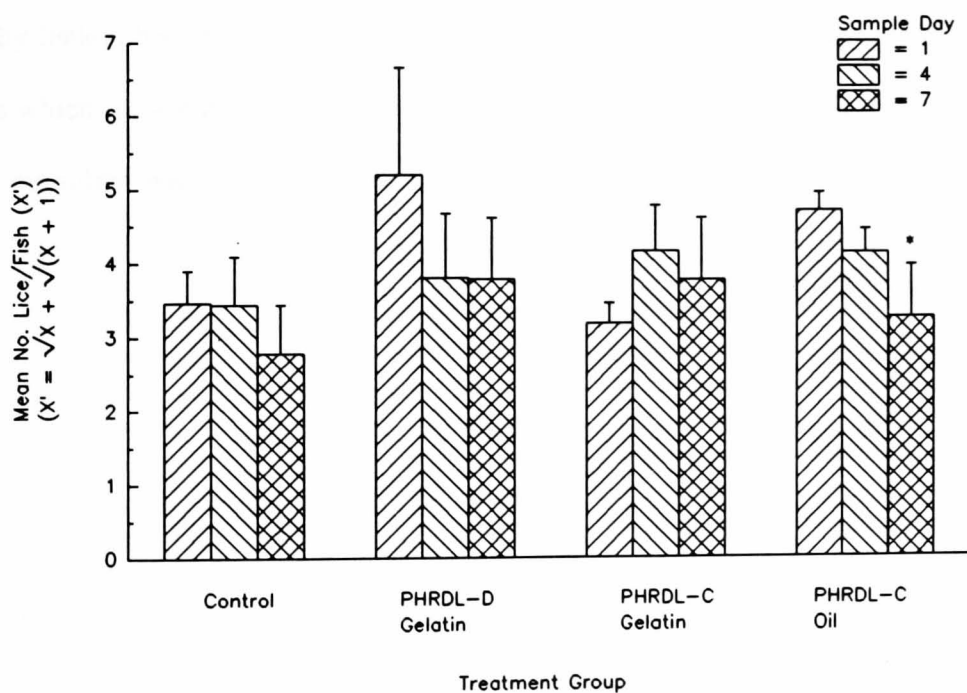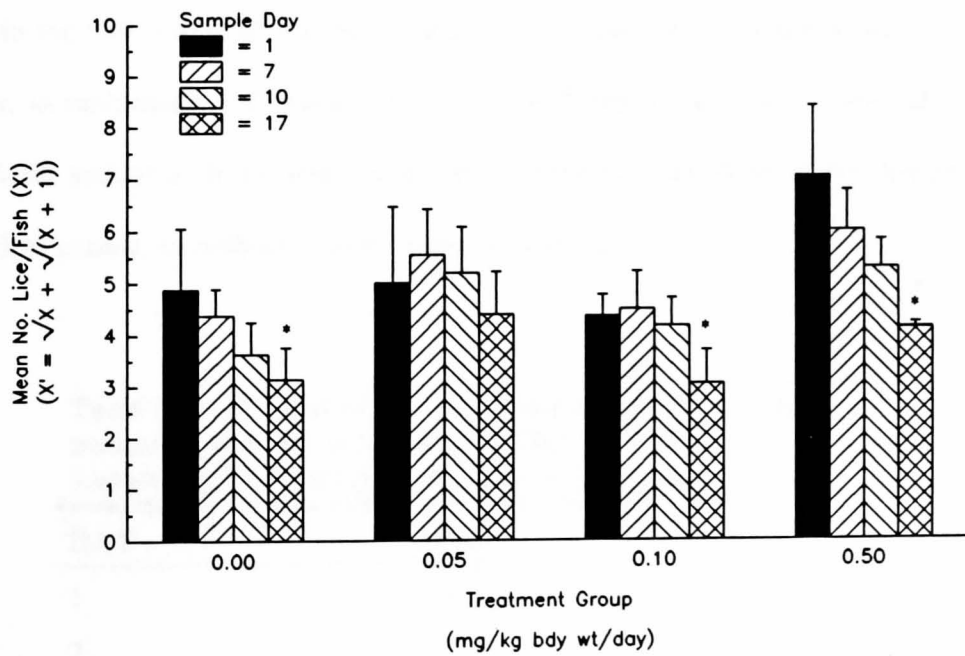**Figure 6.2.** Mean reduction in sea lice (transformed data) infecting Atlantic salmon following oral treatment with PHRDL-D for sample days 1(○) and 2(●)(* = sig. different from control). A, exp. 1; B, exp. 2 (bars = SD).

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Table 6.6. Mean efficacy (% lice reduction from day 1) of Compound PHRDL-D (Experiments 1 & 2) in removing sea lice from lice infected salmon when administered orally.

Dose Rate (mg/kg bdy wt/d)	Mean Efficacy[1]	
	4 Days Post Treatment	7 Days Post Treatment
0.025	45.4	34.9
0.05	45.6	33.7
0.1	42.0[2]	22.9[3]
0.5	51.3	32.3
1.0	68.6	44.7

[1] Means were corrected to reflect lice lost in controls.
[2] Grouped mean for Exp. 1 (54.3%) and Exp. 2 (29.7%).
[3] Grouped mean for Exp. 1 (37.8%) and Exp. 2 (8.0%).

Results from experiment 3 did not support the findings from experiments 1 & 2. Statistical comparisons of the lice numbers within each of the treatment groups indicated that although lice numbers appeared consistent throughout the trial (F = 3.115, P = 0.066), lice numbers were unaffected where fish were treated at 0.1 mg/kg bdy wt/d with either PHRDL-D or PHRDL-C when incorporated into a gelatin binder (PHRDL-D - F = 2.985, P = 0.073; PHRDL-C - F = 2.361, P = 0.120). However, significant reductions in lice were observed in the PHRDL-C treatment (0.1 mg/kg bdy wt/d) when incorporated into the feed with a vegetable oil binder (F = 11.600 P = 0.001) (Figure 6.3).

Notes taken on the feeding behaviour of the fish during experiment 3 indicated that fish in the control, and both gelatin binder diets responded normally to the medicated feed offered, but that the response was not as robust as has been observed in experiments 1 & 2. Fish in the oil binder group responded very poorly to medicated feed offered. One fish mortality was recorded in the PHRDL-D group (sample day 7), believed to be due to handling stress and a bacterial infection.

Figure 6.3. Mean reduction in sea lice (transformed data) infecting Atlantic salmon following oral treatment with PHRDL-C & -D (* significantly different from day 1 counts) (bars = SD).

6.3.3.2   Ivermectin

Results for the treatments with Ivermectin are summarized in Figure 6.4. As stated above, control mortality was significant on day 10 (see Table 6.2). The results from the multiple range tests (Tukey, $P < 0.01$) showed that lice numbers on the controls were stable until day 10 after which numbers were significantly lower. Similarly, lice numbers were significantly lower, with respect to day one counts (Tukey, $P < 0.05$) on day 17 in both of the 0.1 and 0.5 mg/kg bdy wt/d groups. Lice numbers in the 0.05 mg/kg bdy wt/d group were stable throughout the entire experiment ($F = 2.73$, $P = 0.062$).

There were no mortalities in any of the control, 0.05 or 0.1 mg/kg bdy wt/d groups. However, high mortalities were observed in the 0.5 mg/kg bdy wt/d group with 7 (of 10) fish dying before day 7 (2 after day 2, 3 after day 3 and 2 after day 5). Many of the fish in the treatment (0.5 mg/kg bdy wt/d) showed signs of toxicity of the drug as indicated by listless behaviour, immobility and laboured ventilation. In every instance, mortalities which were removed from the group were found to be infected with lice. An eighth mortality was recorded in the 0.5 mg/kg bdy wt/d after the day 10 sampling (day 15).

As stated previously, the greatest
variation with respect to time ...
... (Tukey, $p < 0.05$) the applied ...
were significantly different ...
numbers for each of the overlaps ...
between days 1 and ... ...
chronic loss of ... ...
are given in Table ...
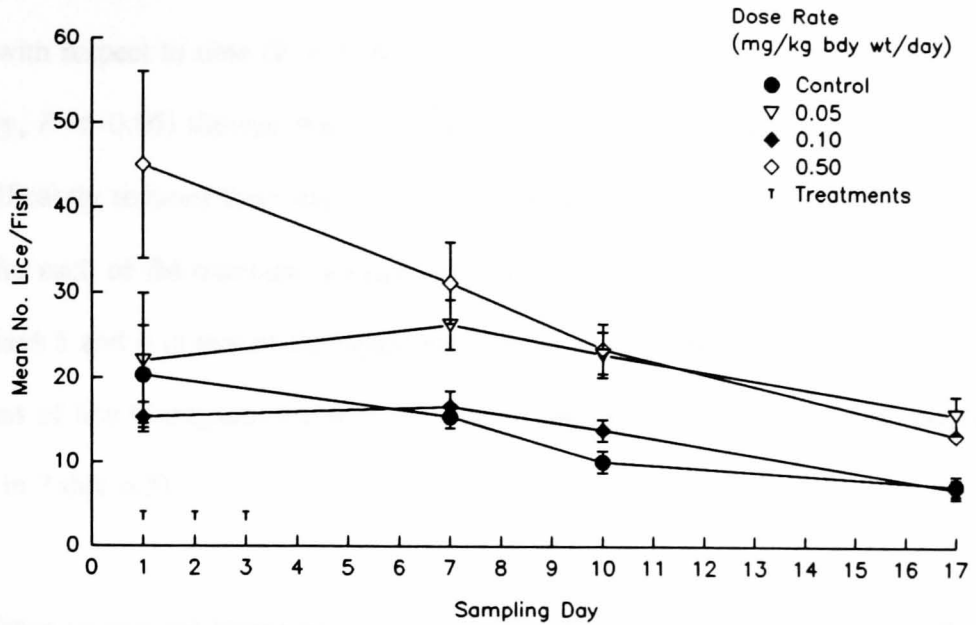
Figure 6.4. Mean reduction in sea lice infecting Atlantic salmon following oral treatment with ivermectin (bars = SD).

Figure 6.5. Mean reduction in sea lice (transformed data) infecting Atlantic salmon following oral treatment with ivermectin (* = significantly different from day 1 count) (bars = SD).

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6.3.3.3   SKB5 & SKB8

As stated previously, lice numbers in the control group showed a high degree of variation with respect to time (F = 5.882, $P \ll 0.05$). Results from the multiple range test (Tukey, $P < 0.05$) showed that lice numbers were stable between days 1 and 4 but were significantly reduced from day 7 onwards. Multiple range tests of the lice numbers for each of the treatment groups with respect to time showed no differences between days 1 and 4 in any of the treatment groups. Due to the high (laboratory) chronic loss of lice throughout the trial, no further analysis was carried out (lice counts are given in Table 6.5).

No mortalities or unusual behaviours were noted in the control or 0.01 and 0.1 mg/kg bdy wt/d groups. However, high mortalities were observed in both 1.0 mg/kg bdy wt/d groups. A total of 7 fish died in the SKB5 group (0.5 mg/kg bdy wt/d) and a total of 8 fish died in the SKB8 group (0.5 mg/kg bdy wt/d). In both cases all fish were lost in the first 4 days of the trial (Table 6.7). Similar to the findings with ivermectin, in each instance mortalities removed were infected with lice. Signs of toxicosis were similar to those observed in fish exposed to lethal doses of ivermectin, including listlessness, immobility and laboured ventilating.

Table 6.7. Number of fish mortalities in fish given oral treatments with the milbemycins SKB5 and SKB8. Sample day 1 = first of three days on medication.

DAY	SKB5	SKB8
1	0	0
2	2	3
3	4	5
4	1	0

6.4   Discussion

The high toxicity of the avermectin, ivermectin, and the structurally similar milbemycin compounds (Fisher 1985) SKB5 and SKB8, to fish, yet lack of effect to lice, when administered orally, is very difficult to explain and may represent limitations of overall laboratory model used. Paradoxically, PHRDL-D, representative of the pyrethroid insecticides which are, in general, considered to be highly toxic to fish (Clark, Goodman, Borthwick, Patrick, Cripe, Moody, Moore & Lores 1989; Haya 1989), was found to be highly efficacious in removing lice, but showed little toxicity towards fish.

The most consistent effect observed throughout the trial work was the chronic loss of lice from control fish. It is possible that due to lack of recruitment from infective copepodids, or adult lice (Bruno & Stone 1990), natural mortality in the lice populations used to infect fish resulted in decreases in infection intensity over time. Alternatively, handling stress, and/or sublethal effects from repeated benzocaine exposure may have contributed to losses of lice. However, previous work (see Chapter 5) showed that benzocaine has little, if any, effect on lice at the concentrations used to anaesthetize fish, though to date there have been no definitive experiments on the toxicity of benzocaine to lice. Other factors which may have contributed to the decrease in lice numbers, such as size and behaviour of the fish, are more difficult to assess, but underlie the difficulties in using artificial conditions as produced in the laboratory to evaluate clinical pharmacy. Similarly, differences in the lice populations appeared evident as indicated by the high variation in infection rates achieved.

The high toxicity of ivermectin, SKB5 and SKB8 complements previously published data on the toxicity of ivermectin to fish. In their preliminary trials on the efficacy of

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oral ivermectin treatment for the control of sea lice, Palmer *et al.* (1987) found that a dose rate of 0.2 mg/kg bdy wt/d was highly efficacious at removing lice but that a dose rate of 0.4 mg/kg bdy wt/d resulted in mortality and lethargy. In contrast, O'Halloran *et al.* (1992) noted elevated mortality and lethargy in salmon smolts treated at 0.2 mg/kg bdy wt/day for the ergasilid copepod *Ergasilus labracis*. At similar dose rates (0.2 mg/kg bdy wt) mortality was also noted in mottled sculpins (*Cottus bairdi*) (intra-muscular injection) (Heckman 1985) as well as eels (*Anguilla anguilla*) (gavage) (Taraschewski *et al.* 1988).

Why a higher rate of mortality was observed by O'Halloran *et al.* (1992) than Palmer *et al.* (1987) under similar experimental conditions is difficult to explain. Size of fish plays a significant role in the toxicity of pesticides in fish (for review see Murty 1986), and in the case of ivermectin, is presumably due to differential relative size of the lipid storage compartment in different size classes of fish (Tarr, Barron & Hayton 1990). Using autoradiographic and scintillation counting techniques with tritium labelled ivermectin, Høy *et al.* (1990) found that peak concentrations of radio labelled material were found primarily in fat containing tissues 4 days after administration. However, relatively high amounts of material were also found associated with nervous tissues, leaving Hoy *et al.* (1990) to conclude that not only is the compound sequestered by lipid containing tissues but that it readily crosses the brain blood barrier, thus accounting for its high toxicity.

Unexpected mortality has been reported following oral ivermectin treatment when used during (cold) winter months (P. Smith pers. comm.). Why ivermectin might be more toxic at lower temperature is unclear, but may be related to slower degradation in the

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tissues, in which under modest temperatures (7.0°C) it persists for up to 28 days (Høy et al. 1990). One might speculate that decreased metabolic rates may lead to the build up of toxic levels in fat or the central nervous system. Since ivermectin has a relatively narrow therapeutic margin, differential feeding responses with experimental groups was believed, in both of the above mentioned studies, to be related to individual negative side effects (i.e. listlessness) and mortality, underlying the need for wide therapeutic ratios where oral chemotherapy is employed with large animal populations.

The finding that ivermectin or either of the milbemycins did not have any therapeutic effect, even at toxic levels, is very difficult to explain, and may be attributed to laboratory related artifacts. Palmer et al. (1987) reported that following a single application of 0.2 mg/kg bdy wt/d lice numbers were reduced by approximately 50% 1 week from treatment and remained low for a further 2 weeks, after which numbers began to rise. Similarly, O'Halloran (1992) reported that reductions in parasite numbers were observed in as little as 2 days following a single treatment at 0.2 mg/kg bdy wt/d. A somewhat longer response time of 4 days was reported by Hyland and Adams (1987) following a single intra muscular (im) injection to goldfish parasitized by Lernaea sp. (species not given). Thus in order to clarify issues pertaining to uptake and concentrations achieved in target tissues (i.e. skin), further work will be required on the pharmacokinetics of ivermectin metabolism in fish.

The high mortality observed in the higher concentrations in the ivermectin, SKB5 & SKB8 trials may be considered as a negative control confirming that fish were consuming the medicated feed. The lack of response, with respect to lice numbers, in all 3 trials with ivermectin, SKB5 and SKB8, especially at toxic concentration, may

reflect the physiological status of the laboratory acclimated fish. It is possible that the lack of effect may have been related to high levels of stress in the fish, as the effect of ivermectin has been shown to be, in part, modulated by the host immune system (Bennett, Williams & Dave 1988). Alternatively, relatively low lipid levels may inhibit the ability of the compound to distribute to necessary tissues. Poor physiological status of the lice used for infecting fish may also have obscured any chemotherapeutic effect. Although such hypotheses are highly speculative, they underlie the limitations of studying chemotherapy under artificial conditions and the need for replicate field testing.

Present data on the therapeutic effect of ivermectin for the treatment of parasites suggest that small dose rates over a protracted treatment period are necessary in order to avoid toxic effects in treated hosts. Thus O'Halloran *et al.* (1992) reported that a dose rate of 0.05 mg/kg bdy wt/d given orally twice a week (time course not given) resulted in significant reductions of lice without any ill effects to fish. Further work by researchers in Ireland have found that the compound can be effective at a range of dose rates and feeding regimes ranging from 0.2 mg/kg bdy wt/d (once every two weeks for several months) to 0.05 mg/kg bdy wt/d (given twice/week; for several months) (Smith *et al.* in press).

Unlike the responses observed with ivermectin, SKB5 and SKB8, the pyrethroid compound PHRDL-D was found to significantly reduce lice numbers within a few days of treatment. At present, there is no available information on the oral uptake of pyrethroids in fish. In general, pyrethroids are considered to be highly toxic to fish (Clark *et al.* 1989; Haya 1989) Why pyrethroids are more toxic to fish than mammals,

which are much more tolerant by several orders of magnitude, is unclear. Although the primary route of elimination is via the bile (Bradbury *et al.* 1986), evidence suggests that very little metabolism by ester hydrolysis, the primary mode of detoxification in mammals (Kaneko, Ohkawa & Miyamoto 1981) occurs in fishes. Similarly, detoxification rates by mixed function oxidases appears to be much lower in trout than mice (Glickman *et al.* 1982). Thus the higher toxicity of pyrethroids to fish appears to be related to slower, or lack of, metabolism resulting in longer retention times in fishes, but may also be related to target site specificity (Glickman *et al.* 1982; Glickman & Lech 1982).

Given the disparity between the results obtained with the pyrethroid and the avermectins/milbemycins, it is difficult to estimate whether the effects observed on the lice were due to ingestion of the compound, unmetabolized, from the fish or due to excreted, unmetabolized, compound acting topically on the lice. In general pyrethroids, absorbed via the gills, concentrate in fat tissues (Bradbury *et al.* 1986; Glickman *et al.* 1981). In both of these studies, the majority of the compound studied (fenvalerate and permethrin) was retained in the carcase or bile (80-90%) with relatively little compound found in the urine, faeces or blood. Estimated half lives for the elimination of pyrethroids in trout are relatively quick (1-2 days) (Glickman *et al.* 1981; Bradbury *et al.* 1986). Although these elimination rates are considerable slower than mammals (6-12 h) (Ohkawa, Kaneko, Tsuji & Miyamoto 1979; Kaneko *et al.* 1981; Glickman *et al.* 1981), they indicate that a substantial proportion of unmetabolized compound is either present in fish tissue immediately after exposure or that a significant amount of compound may be released into the water within a few days of treatment, due to poor absorption capacity (Høy & Horsberg 1990) which may

explain the relatively rapid response seen in experiments 1 & 2 with PHRDL-C. However, the data would also suggest that the therapeutic effect would be short lived as residues would not persist in fish tissues.

As outlined above, it is possible that rapidly excreted compound was acting topically on the lice. However, this seems unlikely as pyrethroids, in general, have high organic binding coefficients resulting in decreased bioavailability (Clark *et al.* 1989; Chandler 1990). As unmetabolized compound is passed through the gut, it is likely that a significant proportion of the ingested dose is passed bound to stomach contents. Results from preliminary trials (not reported here) did reveal that when PHRDL-C mixed in oil was given as a gavage, regurgitated compound significantly reduced lice numbers on experimental fish. Although these trials are not discussed in detail, they did indicate the potential for the compound to act topically in the water column.

The differences observed in efficacy between the PHRDL-D experiments 1 and 2, with respect to the 0.1 mg/kg bdy wt/d treatment groups, probably reflect differential feeding rates in the fish. As discussed earlier, toxicity and negative side effects following oral administration of neurotoxins was believed to be due to overdosing as a result of individual differential feeding rates (Brandal & Egidius 1977; Palmer *et al.* 1987). It is thought that the lack of response observed in the last series of trials was due to a poor feeding response in the fish. The finding that oil coated pellets resulted in significant reductions in lice may be due to the oil/compound mixture separating from the pellet in the water column and acting topically on the lice given the high efficacy demonstrated previously for this compound when used as a bath treatment (see Chapter 5).

The need for isolated lice treatments is necessary when one considers that the selection of resistant pest populations is directly related to the frequency of treatment (Barton 1983; Martin, Anderson, Lwin, Nelson & Morgan 1984; Roush, Hoy Ferro & Tingey 1990). Furthermore the persistence of sub-lethal concentrations in target tissues has also been identified as a major contributing factor to the development of resistance (McKenzie & Whitten 1982). Resistance to ivermectin in *Haemonchus contortus* populations in sheep has developed simultaneously on many continents and in some instances has appeared following as few as three drug treatments (Van Wyk, Malan, Gerber & Alves 1987; Van Wyk & Malan 1988; Echevarria & Trindade 1989; Craig & Miller 1990). Since the efficacy of ivermectin depends on biweekly or bimonthly administration for periods as long as 10 months (Smith *et al.* in press) and can persist in an active form at low concentrations for up to 28 days (Høy *et al.* 1990), the development of resistance in this situation is highly probable (Scott, Roush & Liu 1991). The development of resistance to ivermectin would not only put further restrictions on the potential use of presently licensed chemotherapeutants for the control of sea lice, but could also inhibit the development of much needed alternative chemotherapeutants.

7 Toxic and Sub-lethal Effects of Alternative Chemotherapeutants to Chalimus Stages of the Salmon Louse, *Lepeophtheirus salmonis*

7.1 Introduction

Although the clinical control of sea lice infestations relies primarily on the use of chemotherapeutic agents (Brandal & Egidius 1979; Wootten *et al.* 1982; Reyes & Bravo 1983), treatments are only partially effective due to the selective toxicity of some compounds, such as DDVP and trichlorfon, to adult and preadult stages of lice (Wootten *et al.* 1982). Consequently, there is little, if any, data on the effects of pesticides on larval stages of lice present alongside adults and preadults on host fish. Jakobsen and Holm (1990) reported a 75% reduction in chalimus/fish between treatment and control groups following topical treatments with an oil based pyrethrum formulation. Toxicity to larval lice has also been reported for ivermectin (Palmer *et al.* 1987) and diflubenzuron (Høy & Horsberg 1991) following oral administration to lice infected salmon. However, in all of the above mentioned studies the extent of the toxic effect was not documented.

The implications of larval toxicity extends to several areas with respect to pest management. The first of these is the need for repetitive treatments (Brandal & Egidius 1979; Wootten *et al.* 1982). If larval stages of lice were effectively killed during treatments, the time between treatments would be greatly increased. Secondly, exposure of larval lice to pesticides which are not killing them, but may be having sublethal effects, may promote, or accelerate, the development of resistance (Coles & Roush 1992).

In order to better understand the effects of pesticides on larval lice, a series of experiments was developed to assess acute and subacute toxicity under laboratory conditions. However, to standardize infections with respect to lice development and fish stock, a laboratory *in situ* infection model was developed. In addition to data on the acute toxicity of pesticides to larval lice, the system allowed documentation of larval development with respect to generation time.

7.2   Methods

7.2.1   General

Atlantic salmon (*Salmo salar*) smolts used for the development and toxicity trials were obtained from sea water acclimated stock from the Dunstaffnage Marine Laboratory (Howietoun stock) and from Marine Aquaculture Ltd. (Loch Fyne). Smolts were transferred to holding tanks at the Sea Life Centre (Oban) and allowed to acclimatize for a period of several weeks. During the acclimation period, fish were offered pelleted feed *ad libitum*.

For individual experiments, smolts were infected with laboratory reared *Lepeophtheirus salmonis* copepodid stages. Lice used for the development and toxicity studies were collected from Atlantic salmon from one of several salmon on-growing sites located on the West Coast of Scotland, depending on the availability of suitable fish. Lice were collected as described previously. Groups of lice were placed in beakers of fresh sea water and allowed to incubate for several days, thereby allowing the eggs to hatch and the resultant nauplii to moult into the infective copepodid stage. After a suitable incubation period (5-6 days, dependant on temperature) the contents of several beakers were concentrated into 1 L and enumerated by taking 10 random 5.0 mL aliquots and

counting the number of live and dead copepodids in a Bogorov chamber.

To infect fish, copepodids were added to a holding tank (volume temporarily reduced from 540 to 200 L) containing smolts and allowed to stand static, with aeration, for 4 hours. Tanks used for infecting and holding fish were those at the Sea Life Centre in Oban. After the 4 hour infection period, water flow to the tank was reinstated.

7.2.2 Development Study

To study the development of lice on fish over time, a single group of 43 smolts was infected with lice at a rate of 95 copepodids per fish. Fish were examined by randomly selecting three fish from the group daily, mildly sedating them (0.01 % benzocaine solution) and examining them in a shallow water bath under a low power microscope. Sea water used in the water bath was periodically changed to maintain adequate oxygenation and ambient temperature. After examination, fish were returned to the holding tank. On alternate days, one fish from the group was sacrificed to obtain a sample of lice for reference. The developmental stage and survivability (i.e. alive vs dead) of each louse was recorded. Fish were examined for a total of 53 days, after which the experiment was terminated.

7.2.3 Chalimus Toxicity Studies

Fish were treated with a range of compounds, representing several different classes of pesticides. Compounds used for the chalimus toxicity trials included azamethiphos (50% w/w), resmethrin (20% methanol suspension (MS) & 1% emulsified concentrate (EC)), ivermectin (1% EC) and SKB7 (technical, solubilized in ETOH). For each pesticide tested, fish were given both bath and intraperitoneal (IP) injections in separate

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experiments. Details pertaining to concentrations, expressed in terms of active ingredient, used for each of the treatments are given below.

For treatments, fish were transferred to a separate set of holding tanks and randomly divided into groups of 3 or 5. The tanks used, somewhat smaller than the previously mentioned holding tanks at the Sea Life Centre, hold approximately 25 L and are supplied with flow through sea water from Loch Creran. For bath treatments, volumes were adjusted to 10 L and allowed to stand static for 1 hour with the appropriate amount of test compound added. Following treatment, tanks were flushed and the water supplies to the tanks reinstated. Aeration was maintained in the tanks throughout all experiments. For IP injections, fish were anaesthetized in a mild benzocaine solution (1 mL benzocaine[10] per 1 L sea water) and injected posteriorly, adjacent to the vent, with 100 μL of test compound. Concentrations of injected compounds were adjusted to correspond to the appropriate target concentration (mg/kg) based on sample weights obtained from a sub-sample of fish taken prior to injection. To maintain sterility of injected preparations, autoclaved distilled water was used to dilute pesticides. Supplied pesticide formulations were checked for sterility by swabbing onto tryptone soya agar (TSA) (-/+ NaCl enriched), maintained in sterile glass containers and diluted into sterile distilled water aseptically.

Prior to each experiment, a sample of fish was taken and the fish sacrificed to establish the developmental state of the lice, these fish being referred to, in the following text, as the 'pre-treatment' group. Remaining fish were divided as described above and subjected to the various treatments. Lice were counted 24 hours following treatments,

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[10] 10% in acetone

by anaesthetizing fish in a mild benzocaine solution (as above) and examining them under a low power microscope (as above) to identify the stage and condition (alive vs dead) of larval lice present. Following examination, fish were then returned to their respective holding tanks. Following a variable incubation period, to allow development of attached larval stages (time allowed for each experiment is given below), fish were examined for lice a second time. For secondary examinations, fish were sacrificed and examined as described above.

7.2.3.1   Azamethiphos

Fish for bath treatments were infected at a rate of 134 copepodids/fish. Fish were subdivided into 6 groups of 5 and treated at the following concentrations: pre-treatment, 0.0 (control), 0.1, 0.25, 0.5, 0.75 and 1.0 mg/L. Lice were counted 1 day following treatment and 11 days post treatment.

For IP injections, fish were infected at a rate of 100 copepodids/fish and subsequently divided into 6 groups of 5. Fish were treated at the following dose rates: pre-treatment, 0.0 (control), 0.01, 0.025, 0.05, 0.075 & 0.1 mg/kg. Lice were counted 3 days following treatment and, due to a complete lack of a significant response from the lice, a second injection was given. Fish were subsequently examined 6 days following the second injection.

7.2.3.2   Resmethrin

Fish for bath treatments were infected at a rate of 100 copepodids/fish and subsequently divided in 6 groups of 3. Treatment concentrations included: pre-treatment, 0.0 (control), 0.05, 0.5 & 1.0 (EC); 0.5 & 1.0 (MS) mg/L. Fish were examined for lice 1

and 5 days post treatment.

For IP injections, fish were infected with lice at a rate of 85 copepodids/fish and subsequently divided into 5 groups of 3 and 1 group of 2. Dose rates tested were: pre-treatment, 0.0 (control), 0.2, 0.5, 1.0, 2.0 and 4.0 (2 fish) mg/kg. The MS formulation was used for all the IP injections. Fish were examined for lice 1 and 8 days post treatment.

7.2.3.3   Ivermectin

Fish for the bath treatments were infected at a rate of 206 copepodids/fish and subsequently divided into 6 groups of 3. Fish were treated at the following concentrations: pre-treatment, 0.0 (control), 0.001, 0.01, 0.05, 0.1 & 1.0 mg/L. Fish were examined 1 and 6 days post treatment.

For IP injections, fish were infected with lice at a rate of 97 copepodids/fish and were subsequently divided into 6 groups of 3. Fish were treated at the following dose rates: pre-treatment, 0.0 (control), 0.001, 0.01, 0.05, 0.1 & 0.5 mg/kg. Fish were examined for lice 1 and 7 days post treatment.

7.2.3.4   SKB7

Fish for bath treatments were infected at a rate of 263 copepodids/fish and subsequently subdivided in 5 groups of 3. Treatment concentrations included: pre-treatment, 0.0 (control), 0.01, 0.05, 0.1 and 0.5 mg/L. Fish were examined 1 and 5 days post treatment.

For IP injections, fish were infected a rate of 204 copepodids/fish and subdivided into 5 groups of 3. Fish were injected at the following dose rates: pre-treatment, 0.0 (control), 0.00005, 0.0005, 0.05 and 0.5 mg/kg. Fish were examined 1 and 5 days post treatment.

7.3   Results

7.3.1 Development Study

Temperatures and salinities throughout the trial are given in Figure 7.1. Overall, salinity was slightly more variable than temperature over the experimental period (53 days). Mean temperature was $10.7 \pm 1.5°C$ with a minium of 8.0 and maximum of 13.0°C being recorded. Mean salinity was $33.7 \pm 1.8‰$ with a minimum of 30.0 and maximum of 36.0‰ being recorded. It should be noted that on day 13, a systems failure (due to storm damage) interrupted the water supply to the tank system for 24 hours. During this period water supply to the tanks was shut off, which resulted in a sudden temperature and salinity drop (8.0°C and 30‰) which recovered once the system was back on line.

Eggs were allowed 6 days to incubate, hatch and develop to the copepodid stage before fish were infected. Thus at a temperature of 10.7°C the generation time from egg to gravid female is estimated to be 43 days (Table 7.1). Overall, there was a marked decrease in the population of lice over the course of the experiment. Fish were infected with lice at a rate of 95 copepodids/fish, which resulted in a mean infection intensity of 38.0 copepodids/fish 1 day post infection. By 53 days post infection the infection intensity was 3.4 lice/fish (adult males and gravid females) which corresponded to an overall success rate of 40% 1 day post treatment with a cumulative

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reduction of 91% in the overall number of lice present from 1 day post infection to 53

days post infection (Figure 7.2).
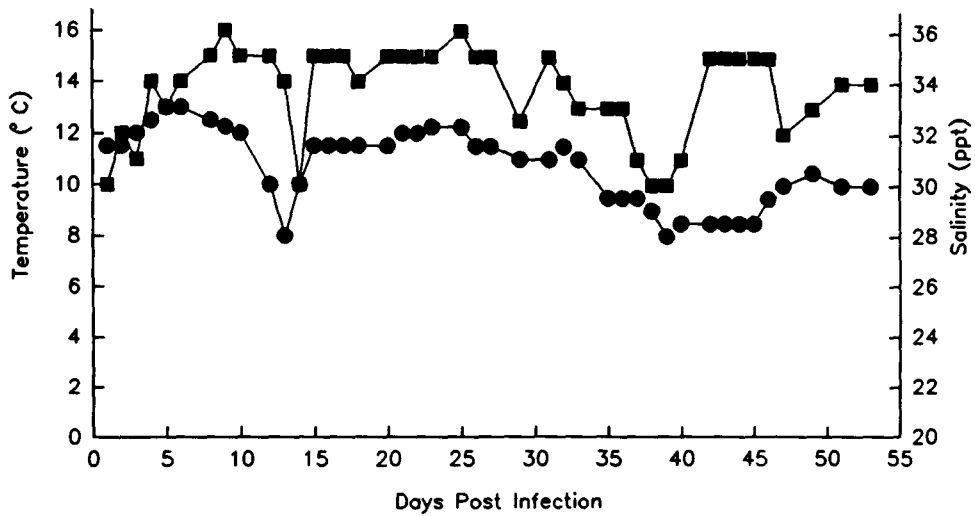
Figure 7.1. Temperature and salinity recordings during laboratory sea lice rearing experiment (● = Temp., ■ = Sal.).
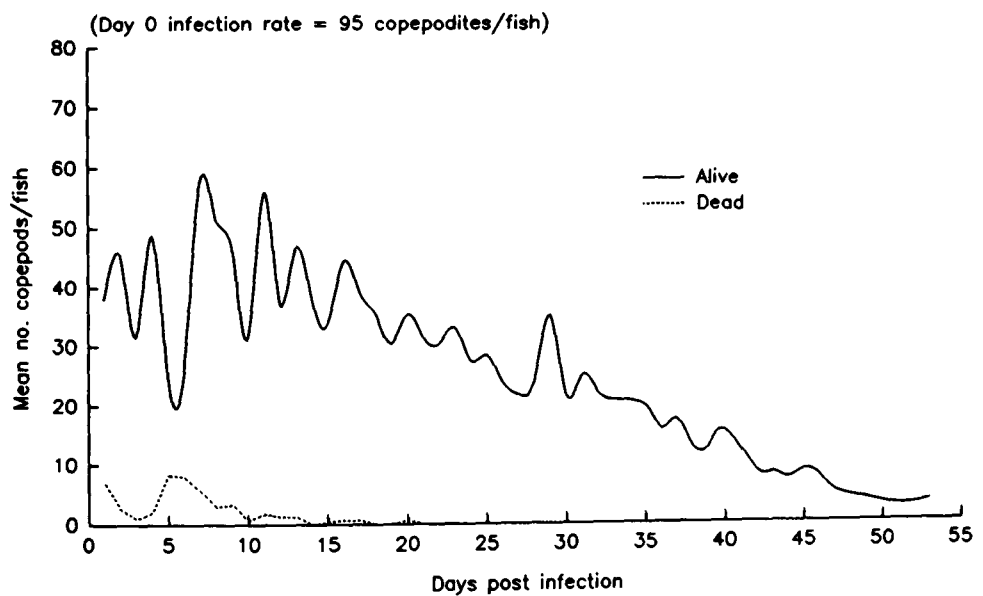
Figure 7.2. Mean number of lice, all stages, alive and dead, per fish (n=3/sample) recorded during laboratory rearing experiment.

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The subsequent developmental times of chalimus larvae, pre-adult and adult lice are summarized in Figure 7.3 and Table 7.1. Based on the time when gravid females were collected (with non-pigmented eggs) the mean cumulative development times (CDT) (defined as the time when 50% of the population represents a particular stage based on a least squares model (Johnson & Albright 1991a)) for each of the developmental stages were (expressed in days): copepodid, 5.0 - 8.4; chalimus 1, 11.1; chalimus 2, 13.2; chalimus 3, 15.3; chalimus 4, 16.4; preadult (male & female), 25.8 and adult (male and female), 31.5 (Table 7.1). However, it was noted that male development was much quicker than female. Gravid females were first recorded 43 days from the onset of the experiment, with individuals with pigmented eggs being observed several days later. Unfortunately attempts at hatching the eggs from the females (*in vitro*) proved unsuccessful. Although the experiment was terminated 53 days post infection, adult males were maintained for 32 days. The duration (time from first appearance to last appearance) of the copepodid and chalimus stages was estimated to be 9-11 days (copepodid), 8 days (chalimus 2), 9 days (chalimus 2), 7 days (chalimus 3) and 9 days (chalimus 4) (Table 7.1).

Moribund or dead lice were only observed on fish for a period of up to 21 days post infection (Figure 7.2 & 7.4). Chalimus stages (i.e. attached) were observed for approximately 19 days. Since preadult and adult lice lose their frontal filament, it is suspected that a substantial proportion of any moribund or dead lice would have been missed during the routine counting. Where moribund lice were observed, mortality rates (all stages) ranged from 0.8 (18 days post infection) to 25.5 % (5 days post infection) (Figure 7.4). In general highest mortality rates were observed during the latter phase of individual developmental stages (Figure 7.4).

Table 7.1. Cumulative developmental time (CDT), time of first and last appearance, and duration of various developmental stages of *Lepeophtheirus salmonis* reared at 10.7 ± 1.5°C (d = days, IE = immature eggs, ME = mature eggs).

Stage	CDT (d)	First appearance (d)	Last appearance (d)	Duration (d)
Nauplius (1/2)	-	1 ≥ 3	-	-
Copepodid	5.0 - 8.4	3 ≥ 5	14	11 - 9
Chalimus 1	11.1	10	18	8
Chalimus 2	13.2	11	20	9
Chalimus 3	15.3	14	21	7
Chalimus 4	16.4	15	24	9
Pre-adult 1	20.4			
♂		18	28	10
♀		20	30	10
Pre-adult 2	25.8			
♂		21	31	10
♀		25	36	11
Adult	31.5			
♂		26	58+	32+
♀		28	55	27+
Gravid ♀-IE		43	58+	15+
Gravid ♀-ME		53	58+	5+
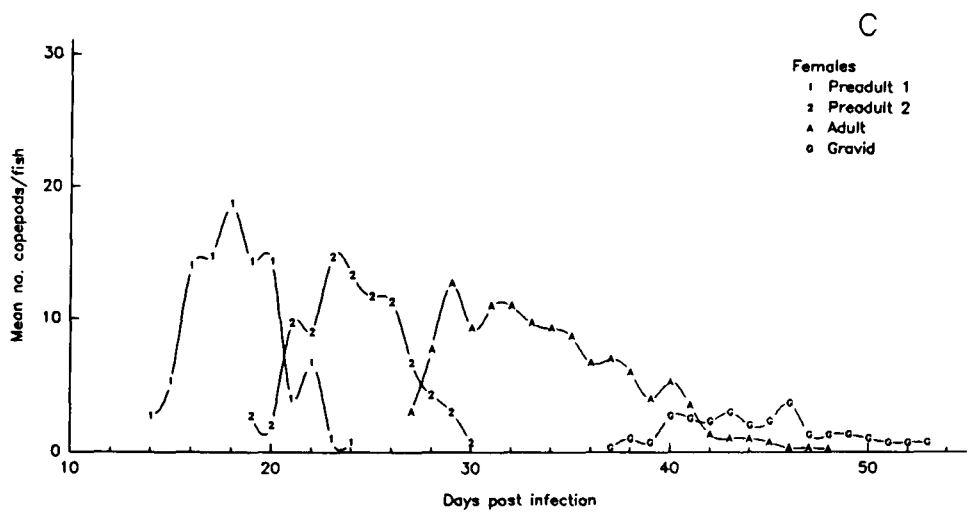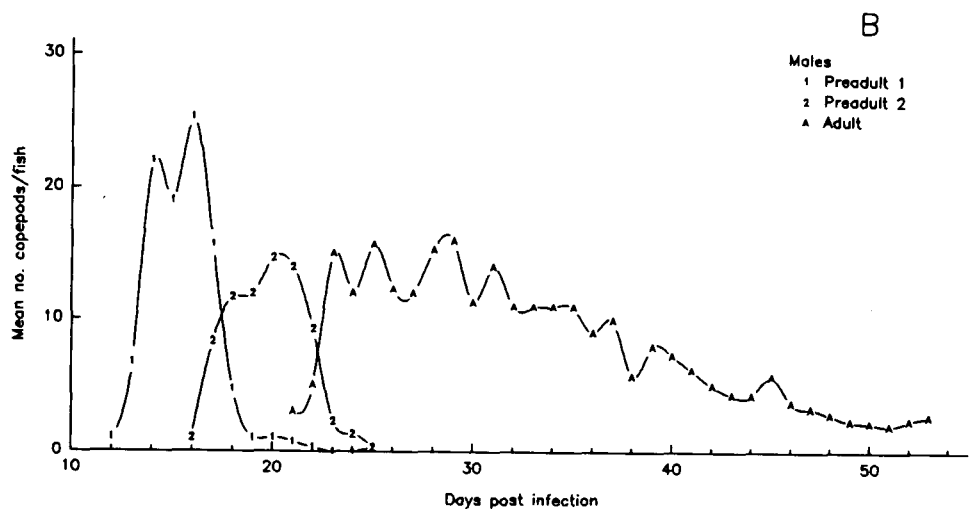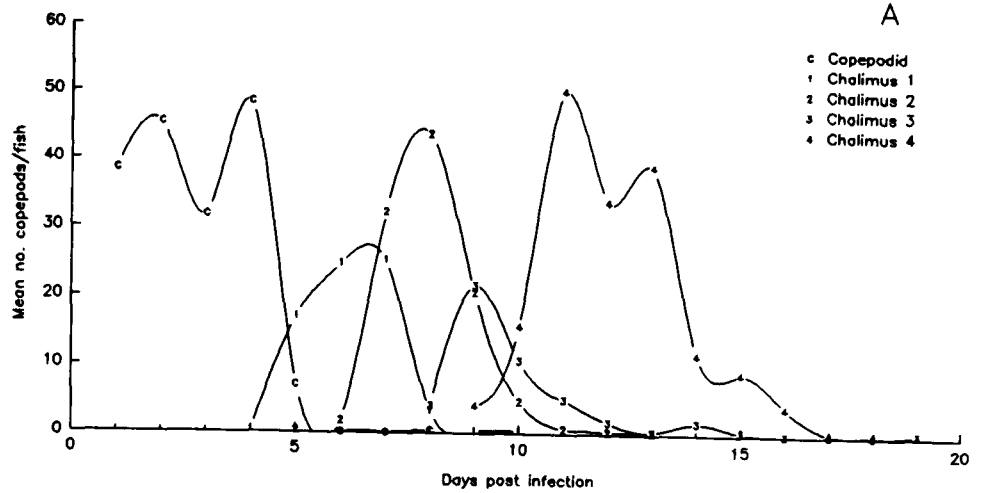
Figure 7.3. Mean number of lice per fish (n = 3/sample) recorded during laboratory rearing experiment (A = copepodid, chalimus 1-4; B = preadult/adult males; C = preadult/adult/gravid females).

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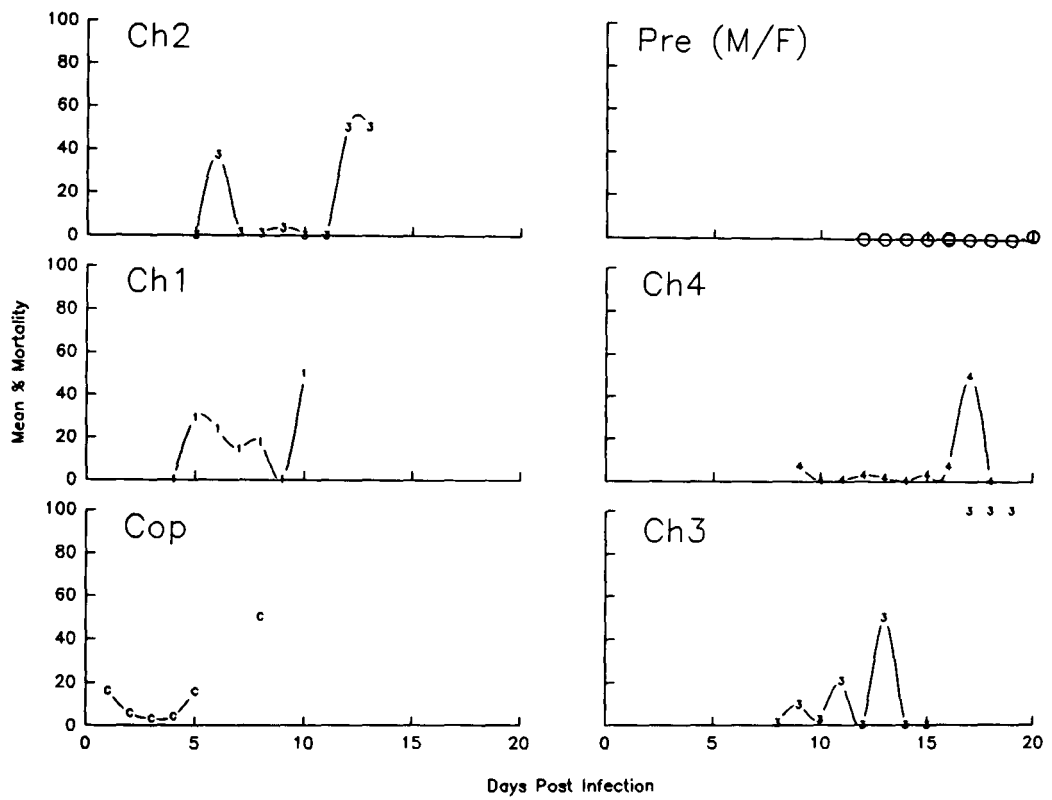
Figure 7.4. Mean % mortality recorded for copepodids, chalimus and preadult lice reared under laboratory conditions (Cop = copepodid; Ch1-4 = chalimus 1-4; Pre = preadult).

7.3.2 Chalimus Toxicity Studies

Temperature, salinity, group size and mean fish weights for each of the chalimus toxicity experiments is summarized in Table 7.2. Overall, temperature throughout the trial period ranged from 12.5 to 14.5°C. An exception was the injection trial with resmethrin which was conducted at a somewhat cooler temperature (11.5°C). The cooler temperature was the result of a systems failure which allowed temperature in the holding tanks to drop as described above. Salinity, much more constant, ranged from approximately 33 to 34‰. Mean weight of fish used throughout the trial ranged from 42.0 to 97.0 g.

Results for various *in vitro* lice cultures are summarized in Figure 7.3a. Although the overall productivity of the system was low, with each female producing 89.1 viable copepodids (38 - 164), mortality rates in the cultures were low (23.1 %) (7.0 - 60.4). On average it was found that approximately 50 females (= 100 egg strings) were required to obtain 4,900 viable copepodids (range: 1,900 - 8,200) (Table 7.3a).

Results for the success of the infections are summarized in Table 7.3b. The overall success of infection was estimated to be 44.2% (18.2 - 71.3) 5/6 days post infection.

Table 7.2. Temperature, salinity, group size and mean fish weight recorded during *in vivo* chalimus toxicity trials.

Compound	Treatment	Temperature (°C)	Salinity (‰)	No. fish /conc.	X Wt. Fish (g)
Azamethiphos	Bath	12.5 ± 2.0	34.0 ± 1.0	5	46.3 ± 6.3
Azamethiphos	Injection	13.0 ± 1.0	34.0 ± 1.0	5	45.6 ± 6.5
Resmethrin	Bath	13.5 ± 0.5	34.5 ± 0.5	3	58.2 ± 14.1
Resmethrin	Injection	11.5 ± 1.5	34.0 ± 1.0	3	97.2 ± 23.4
Ivermectin	Bath	14.5 ± 0.5	33.0 ± 2.0	3	68.2 ± 10.4
Ivermectin	Injection	14.5 ± 0.5	33.0 ± 2.0	3	72.4 ± 13.4
SKB7	Bath	14.0 ± 1.0	33.5 ± 1.0	3	42.9 ± 8.8
SKB7	Injection	14.0 ± 1.0	33.5 ± 1.0	3	65.3 ± 11.8

Table 7.3a. Summary of culture parameters and results for rearing larval sea lice (copepodids) used for laboratory infections (B = Bath treatment; IP = intraperitoneal injection).

Compound/ Treatment	No. ♀'s cultured	Incubation Time (d)	Temp. (°C)	No. copepodids/mL recovered (1000mL)			Estimated number of viable copepodids cultured per female
				Alive	Dead	% Mortality	
Development Study	60	6	12.3 ± 0.2	4.1	1.0	19.6	68.0
Azamethiphos-B	70	9	11.2 ± 0.3	5.0	2.5	33.3	71.4
Azamethiphos-IP	50	7	12.5 ± 0.0	3.2	2.1	39.6	64.0
Resmethrin-B	80	6	14.7 ± 0.6	6.6	1.0	13.2	82.5
Resmethrin-IP	50	6	12.0 ± 1.0	3.7	0.3	7.5	74.0
Ivermectin-B	5	7	13.8 ± 0.1	8.2	1.2	12.8	164.0
Ivermectin-IP	50	7	14.0 ± 0.0	1.9	2.9	60.4	38.0
SKB7-B	50	6	13.9 ± 0.3	6.6	0.5	7.0	132.0
SKB7-IP	50	6	13.5 ± 0.1	5.4	0.9	14.3	108.0

Table 7.3b. Summary of infection parameters and success rate recorded during laboratory development study and for chalimus toxicity studies (B = bath; IP = intraperitoneal injection; cop = copepodids).

Compound-Treatment	No. Fish Infected	Infection Rate (cop./fish)	Temp. (°C)	Incubation Time (d)	Pre Treatment Lice Counts (lice/fish[†])	Success Rate (%)
Development Study	43	95	11.5	1	38.0 ± 13.9	40.0
			12.2 ± 0.5	5	24.3 ± 13.7	25.6
			12.3 ± 0.6	6	26.4 ± 12.3	27.8
Azamethiphos-B	37	135	11.7 ± 0.2	5	27.0 ± 9.0	20.0
Azamethiphos-IP	35	91	13.7 ± 0.3	5	16.6 ± 5.4	18.2
Resmethrin-B	20	100	13.6 ± 0.2	5	60.3 ± 14.6	60.3
Resmethrin-IP	21	81	10.5 ± 1.4	5	22.3 ± 4.3	27.2
Ivermectin-B	20	200	14.0 ± 0.0	6	106.6 ± 25.1	53.3
Ivermectin-IP	20	95	14.3 ± 0.3	5	46.7 ± 3.9	49.2
SKB7-B	20	264	14.1 ± 0.2	5	188.2 ± 33.5	71.3
SKB7-IP	20	204	13.9 ± 0.1	6	143.0 ± 38.8	70.0

[†] all stages combined

7.3.2.1   Azamethiphos

Twenty four hours following bath treatment with azamethiphos there were 7 fish

mortalities: 2 in the 0.5 mg/L group; 3 in the 0.1 mg/L group; 3 in the 0.75 mg/L

group and 2 in the 1.0 mg/L group. Results of the lice counts for the pretreatment

(denoted as "0.00" in the 1 day post treatment figure) controls, 1 and 11 day post

treatment mortalities (lice) following exposure to azamethiphos are plotted in Figure

7.5. One day post treatment, examination of moribund fish indicated a slight trend of

increasing lice mortality with concentration. However, 11 days post treatment, there

were significant numbers of surviving lice, all of which had developed at the same rate

as the controls. A slight reduction in the mean numbers of lice present in the treatment

groups, as compared to the controls, was noted.

Following IP injection with azamethiphos, no fish mortalities were observed. There

was no observable effect on lice mortality or development 3 days following treatment

(one injection) or 6 days following treatment (2nd injection given three days after the

first) with azamethiphos (Figure 7.6).

Azamethiphos - Bath
1 Day Post Treatment

☐ -Cop/Ch1/Ch2
▨ -Ch3/Ch4
▩ -Pre/Adults

Azamethiphos - Bath
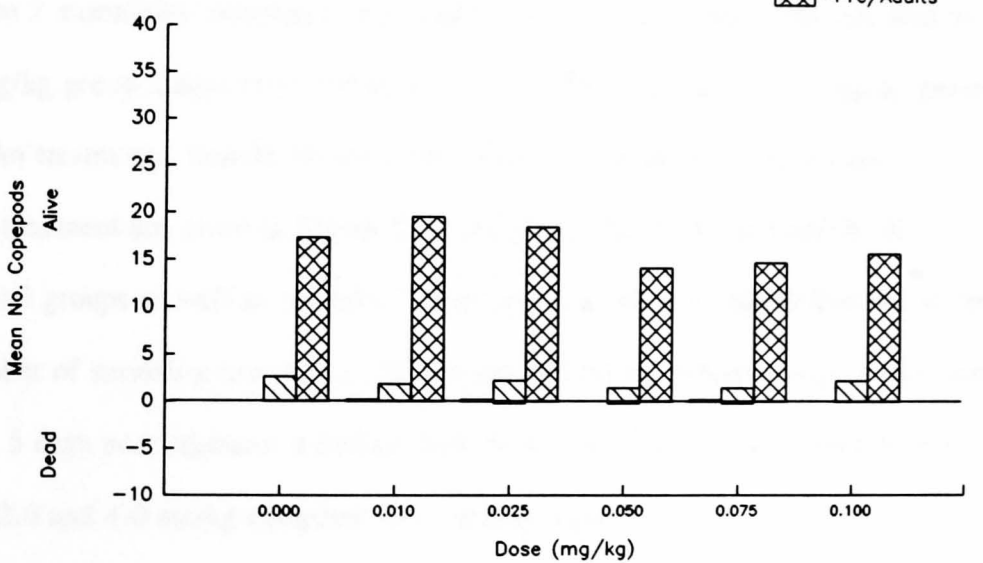11 Days Post Treatment

☐ -Cop/Ch1/Ch2
▨ -Ch3/Ch4
▩ -Pre/Adults
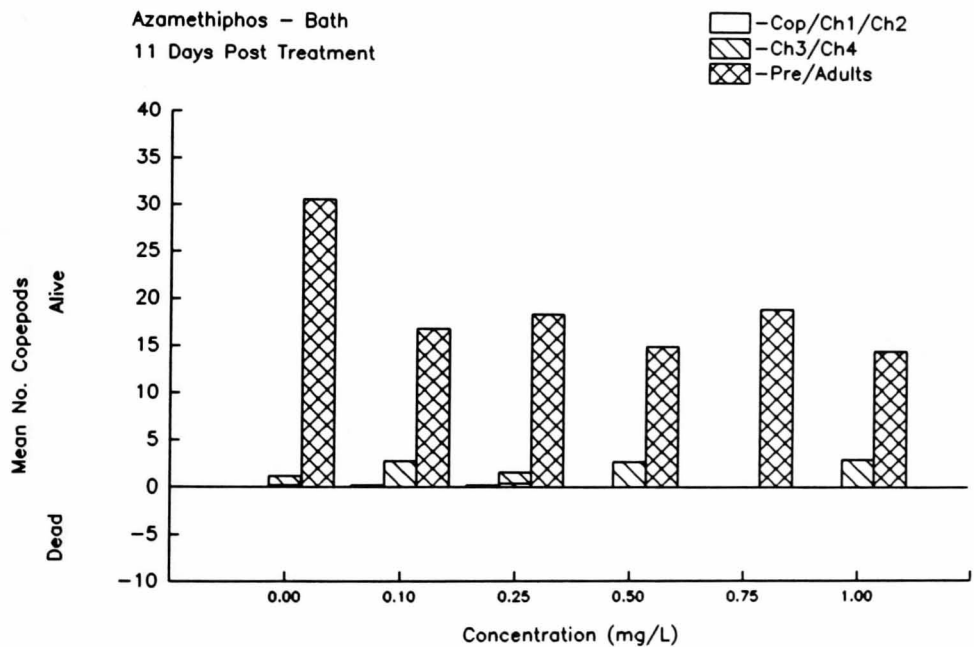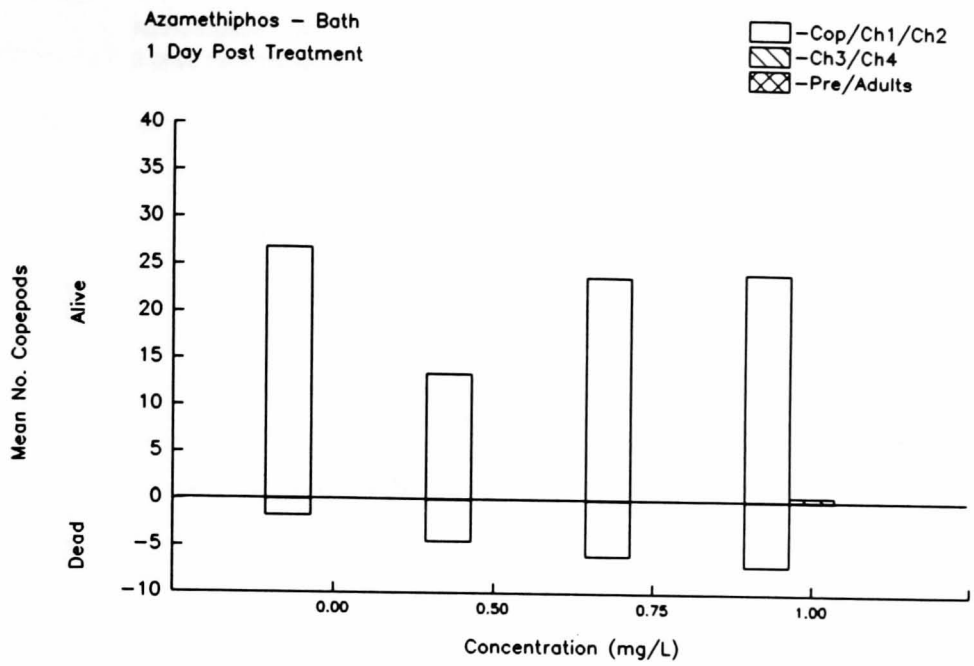
Figure 7.5. Acute toxicity of azamethiphos to sea lice following 1 hour bath exposure to lice infected salmon (Cop = copepodid, Ch1-4 = chalimus 1-4, Pre = preadult).

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Figure 7.6. Acute toxicity of azamethiphos to sea lice following intra-peritoneal injection (IP) to lice infected salmon (Cop = copepodid, Ch1-4 = chalimus 1-4, Pre = preadult).

7.3.2.2   Resmethrin

There were no fish mortalities recorded for any of the sampling points for either of the treatments. However, fish in the 1.0 mg/L (MS) were notably stressed by the end of the treatment as indicated by sporadic coughing and convulsions. The most notable behaviour was sporadic convulsions (head snapping from side to side). Fish in the 1.0 mg/L (EC) group did not display these behaviours. Results for the mean number of parasites following bath treatments, 1 and 5 days post treatment, are given in Figure 7.7. Although few lice mortalities were recorded, there appeared to be a possible effect on development as indicated by a high proportion of early chalimus stages (copepodid, chalimus1 & chalimus 2) to late chalimus stages (chalimus 3 & chalimus 4) 1 day post treatment. However, 5 days post treatment, overall numbers and stages of chalimus present appeared consistent, with the exception of the 0.5 mg/L treatment (MS) which appeared somewhat reduced.

There were 2 mortalities recorded in the resmethrin IP injected fish. One fish died in the 1.0 mg/kg group 2 days from treatment. Another fish died in the 2.0 mg/kg group 8 days from treatment. Results for the mean number of parasites 1 and 8 days following treatment are given in Figure 7.8. Moribund lice were recorded in all experimental groups as well as controls. There appeared to be a slight reduction in the mean number of surviving lice in the 1.0, 2.0 and 4.0 mg/kg treated groups 1 day post injection. 5 days post injection, a distinct lack of lice development was noted in fish treated at 2.0 and 4.0 mg/kg compared to control groups.
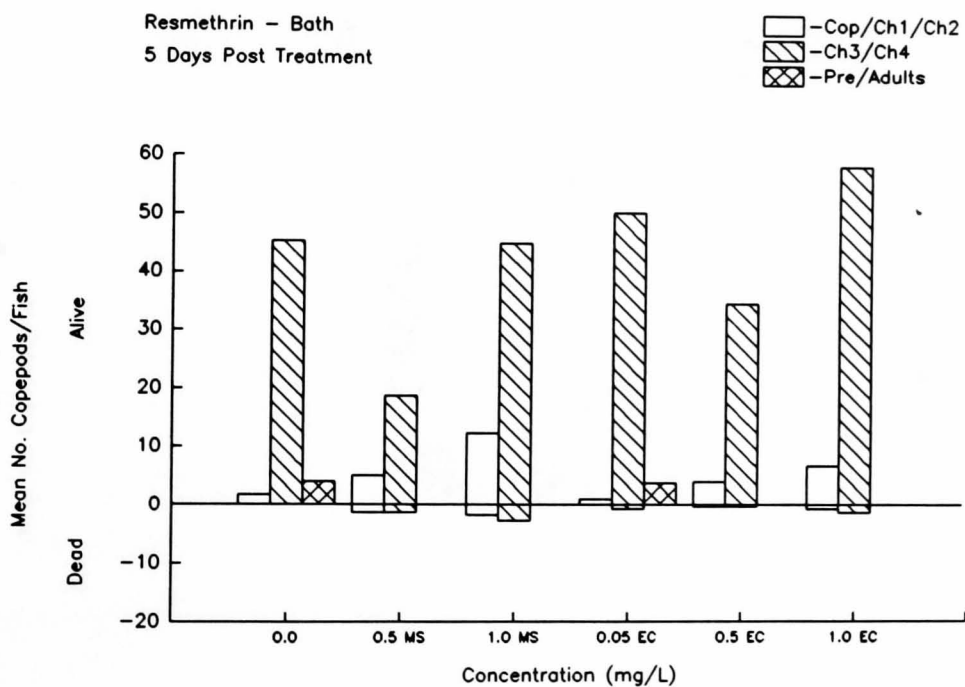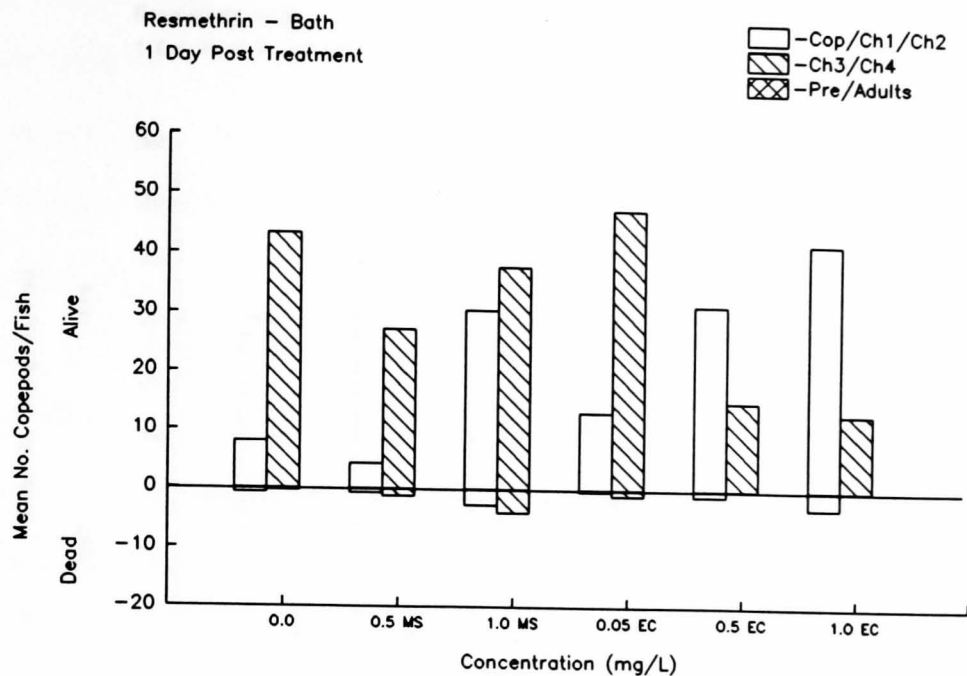
Figure 7.7. Acute toxicity of resmethrin to sea lice following 1 hour bath exposure to lice infected salmon (Cop = copepodid, Ch1-4 = chalimus 1-4, Pre = preadult, MS = methanol suspension, EC = emulsified concentrate).
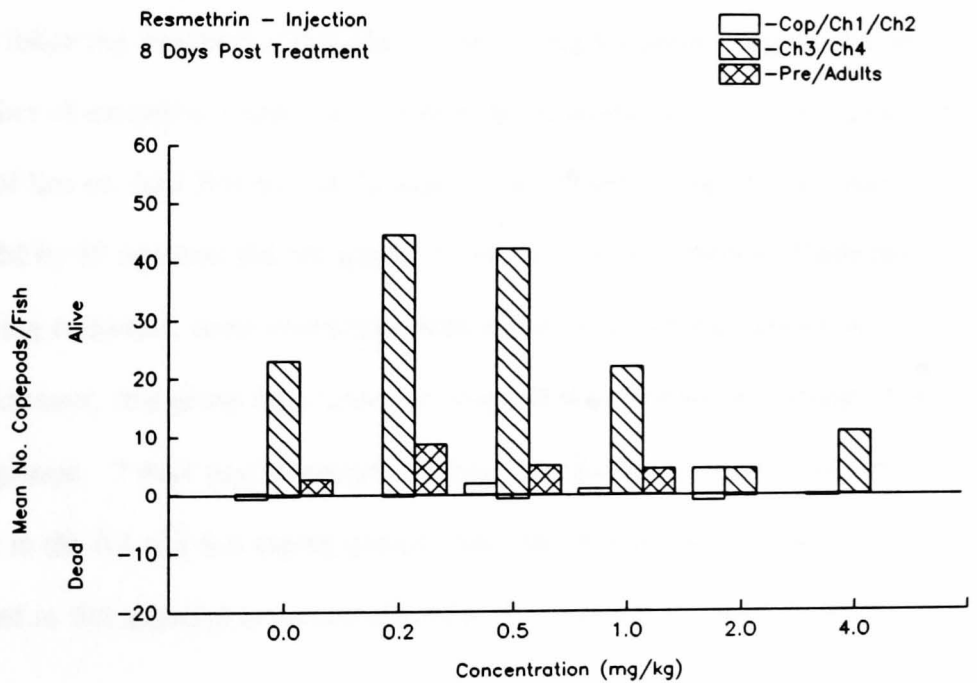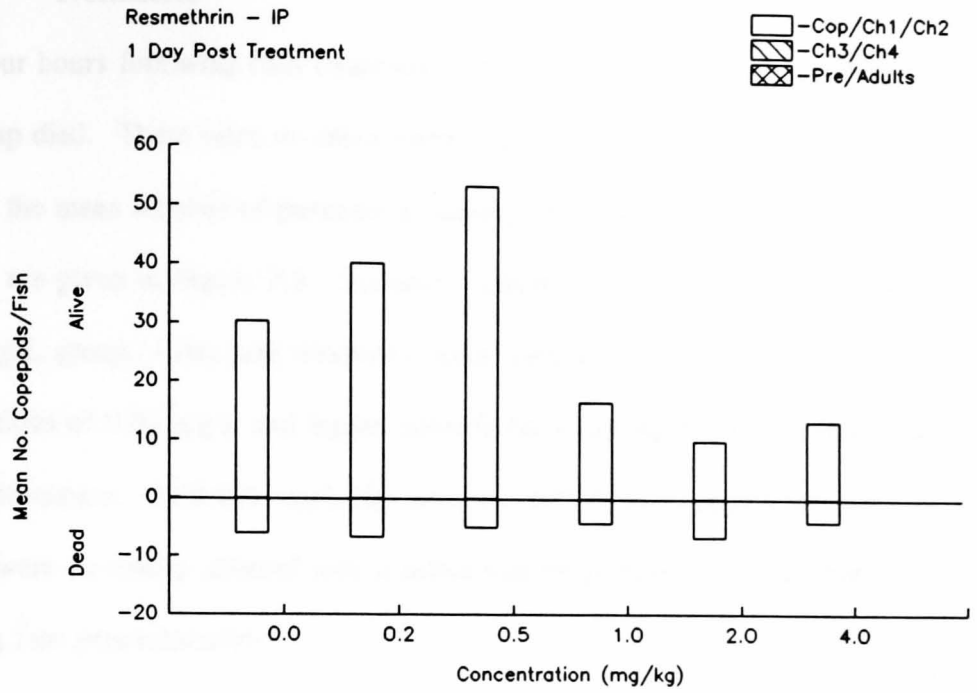
229

Resmethrin – IP
1 Day Post Treatment

☐ –Cop/Ch1/Ch2
▨ –Ch3/Ch4
▩ –Pre/Adults

Resmethrin – Injection
8 Days Post Treatment

☐ –Cop/Ch1/Ch2
▨ –Ch3/Ch4
▩ –Pre/Adults

Figure 7.8. Acute toxicity of resmethrin (MS) to sea lice following intra-peritoneal injection (IP) to lice infected salmon (Cop = copepodid, Ch1-4 = chalimus 1-4, Pre = preadult).

7.3.2.3   Ivermectin

Twenty four hours following bath treatment with ivermectin, all three fish in the 1.0 mg/L group died. There were no other mortalities in any of the remaining groups. Results of the mean number of parasites following treatment, 1 and 6 days post treatment, are given in Figure 7.9. However, values for parasite burdens for the 0.05 and 0.1 mg/L group, 1 day post treatment, were obtained for only 1 fish. Concentrations of 0.01 mg/L and higher were found to be highly toxic to lice 1 day following treatment. At 0.001 mg/L lice were not killed, but 6 days following treatment were adversely affected with a substantial proportion of the lice not developing into preadults/adult.

There were no fish mortalities 1 day following IP injection treatments with ivermectin. Two days following treatment 1 fish died in each of the 0.05 and 0.5 mg/kg groups. Four days following treatment 2 fish died in the 0.1 mg/kg group. Results for the mean number of parasites, 1 and 7 days following treatment, are given in Figure 7.10. Numbers of lice on dead fish are not included in the figures. Ivermectin when administered by IP injection did not appear to be as toxic as immersion treatments. 1 day following treatment, some mortalities were noted in lice on fish treated at 0.5 mg/kg. However, this group also contained preadult/adult lice notably absent in the other test groups. 7 days post treatment, although an absence of acute mortality was noted, lice in the 0.1 and 0.5 mg/kg groups were not developing at the same rate as lice attached to fish exposed to concentrations of 0.05 mg/kg or lower or on control fish.
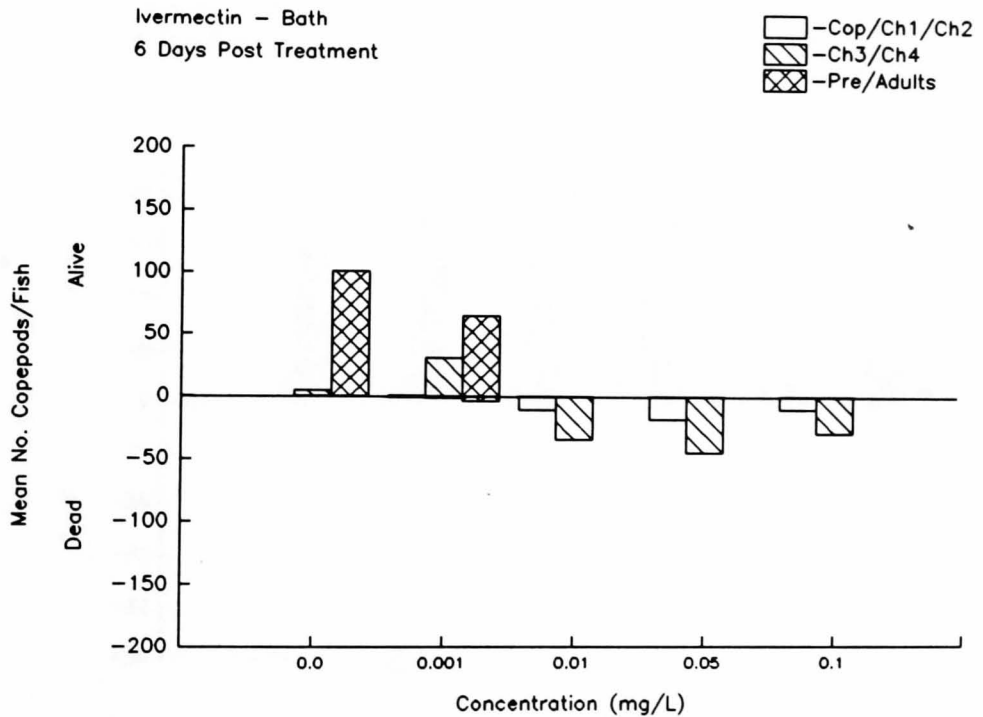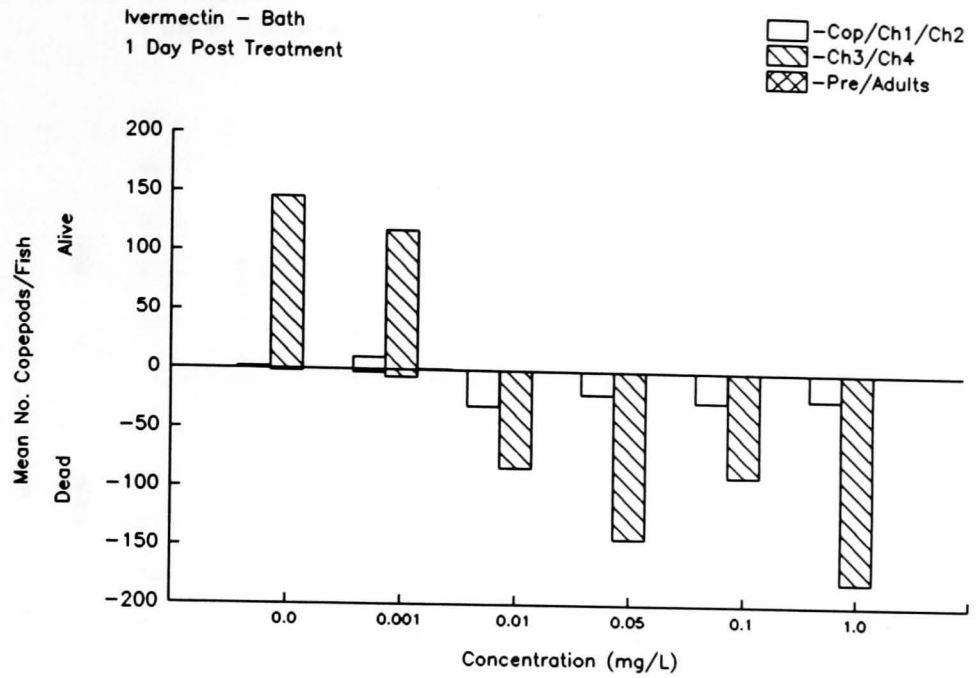
Figure 7.9. Acute toxicity of ivermectin to sea lice following 1 hour bath exposure to lice infected salmon (Cop = copepodid, Ch1-4 = chalimus 1-4, Pre = preadult).
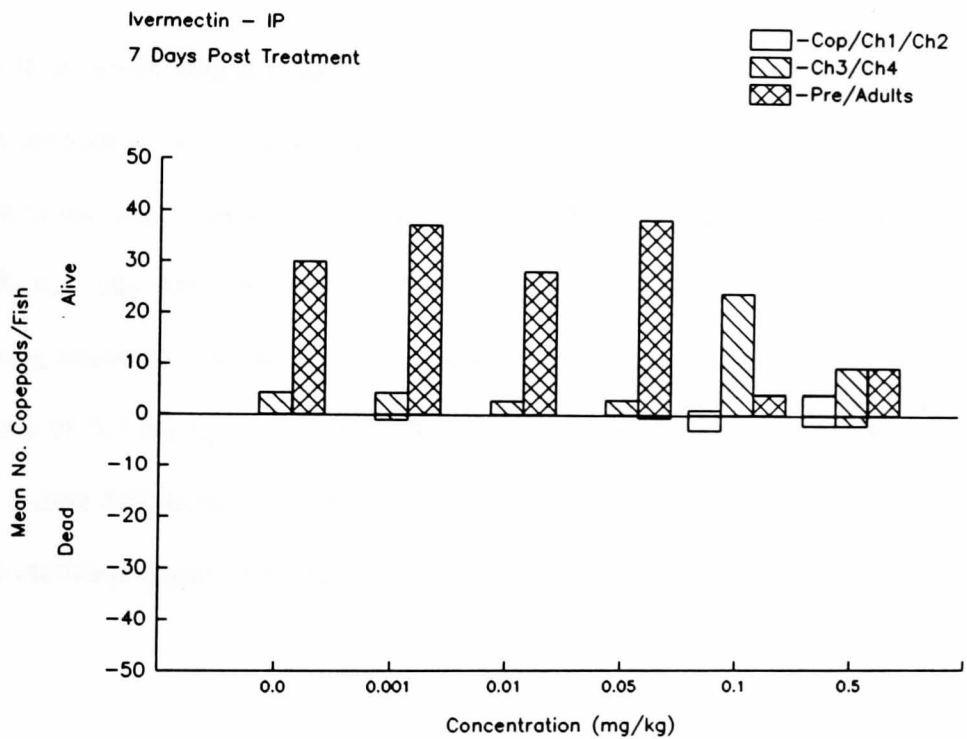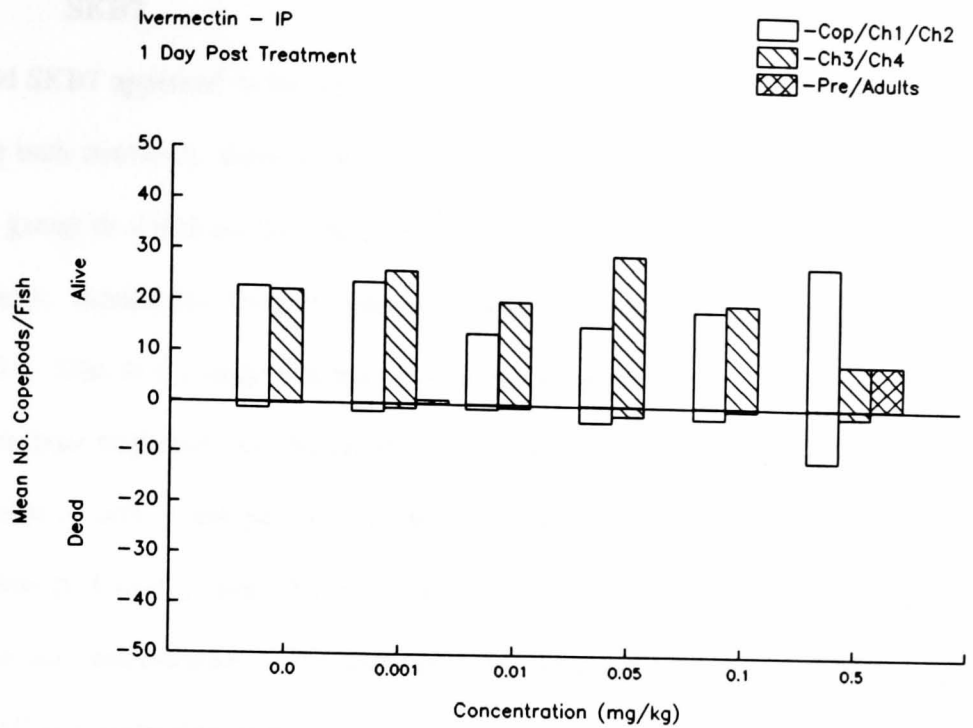
Figure 7.10. Acute toxicity of ivermectin to sea lice following intra-peritoneal injection to lice infected salmon (Cop = copepodid, Ch1-4 = chalimus 1-4, Pre = preadult).

7.3.2.4   SKB7

Compound SKB7 appeared to be the most active compound against larval lice. Following bath treatment, there were no fish mortalities in any of the groups except the 0.5 mg/L group in which all fish were dead within four hours from the beginning of the treatment. Results for the bath treatments with compound SKB7 are plotted in Figure 7.11. Due to the large number of parasites encountered values given in Figure 7.11, 1 day post treatment, for the controls, 0.01, 0.05 and 0.1 mg/L are representative of only 1 fish per concentration. Lice numbers for the remaining concentration (0.5 mg/L), and all concentrations, 5 days post treatment, were obtained from 3 fish per concentration. The compound was found to be highly toxic to larval lice, with all lice appearing dead 1 day following treatment and in the subsequent 5 day post treatment examinations.

Following IP injection with compound SKB7, there were no fish mortalities in any of the groups throughout the treatment period. Results are summarized in Figure 7.12. Again, due to the large numbers of parasites, values for the 0.00005, 0.0005 & 0.005 mg/kg groups, 1 day post treatment, are taken from only 1 fish per concentration. 1 day following treatment, the compound was highly effective at killing lice at a concentration of 0.5 mg/kg. Although 0.05 mg/kg was initially found to be relatively non-toxic, 5 days following treatment, lice in this group indicated that development to subsequent chalimus stages might have been impaired.
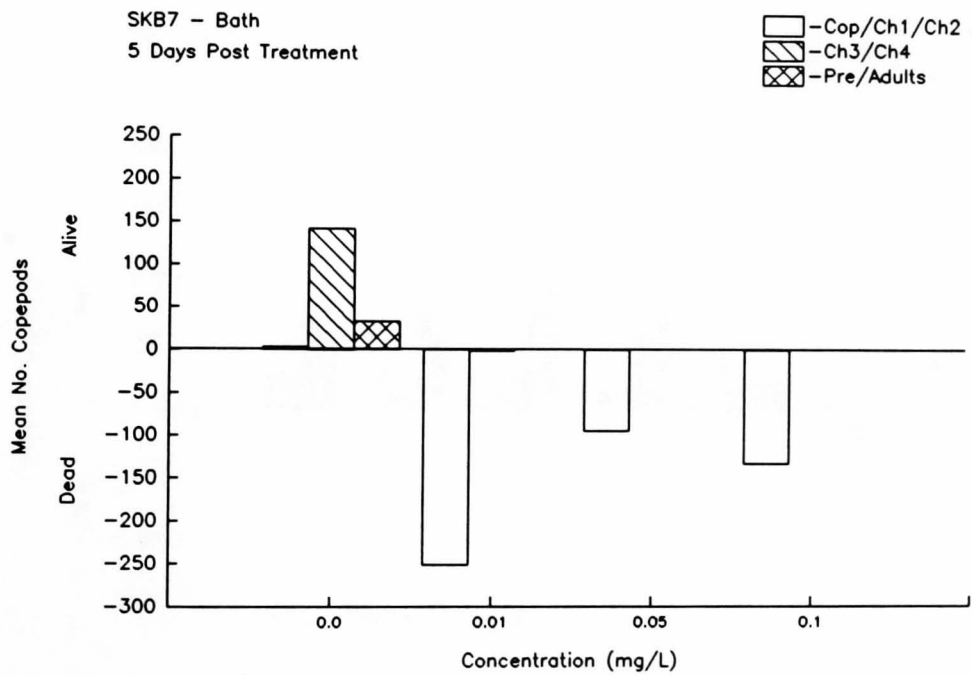
SKB7 – Bath
1 Day Post Treatment

–Cop/Ch1/Ch2
–Ch3/Ch4
–Pre/Adults

SKB7 – Bath
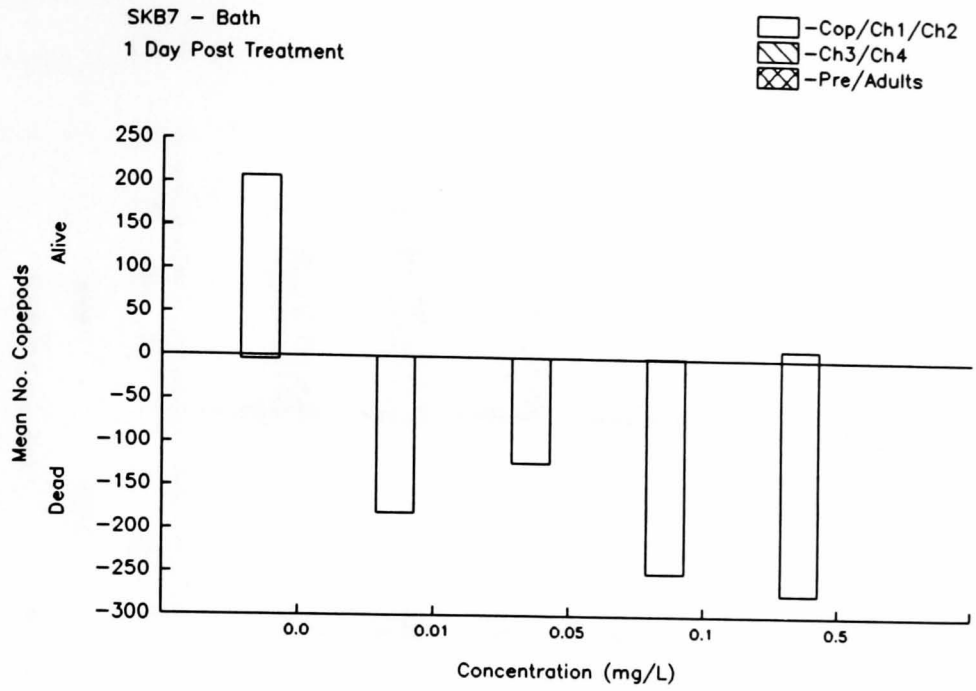5 Days Post Treatment

–Cop/Ch1/Ch2
–Ch3/Ch4
–Pre/Adults

Figure 7.11. Acute toxicity of SKB7 to sea lice following 1 hour bath exposure to lice infected salmon (Cop = copepodid, Ch1-4 = chalimus 1-4, Pre = preadult).
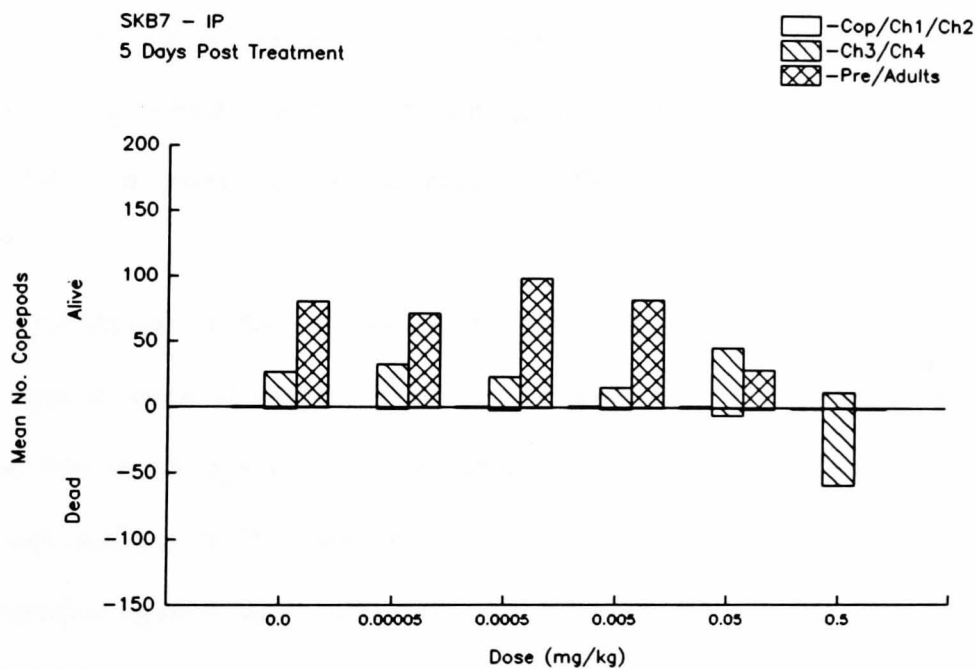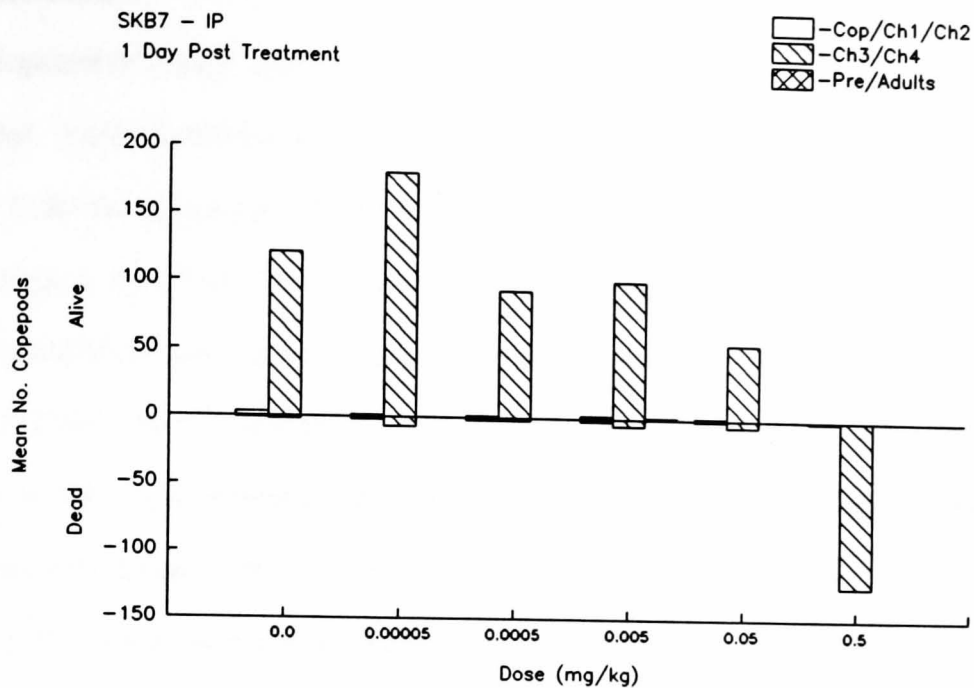
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Figure 7.12. Acute toxicity of SKB7 to sea lice following intra-peritoneal injection (IP) to lice infected salmon (Cop = copepodid, Ch1-4 = chalimus 1-4, Pre = preadult).

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7.4 Discussion

The development of caligid copepods under laboratory conditions has been well documented. For *Lepeophtheirus salmonis*, time to hatch, defined as the time from egg extrusion to the first appearance of the nauplius 1 stage, was calculated to be 5.5, 8.6 and 17.5 days at 15, 10 and 5°C (Johnson & Albright 1991a). In contrast, Johannessen (1978) determined the developmental time to be somewhat longer, estimating the time to hatch at 10-14, 24 and 33-39 days at 11.5, 9.5 and 9.0°C respectively. At higher temperatures, other egg developmental times include: 5.7-6.1 days at 15°C for *L. kareii* (= *L. hospitalis*) (Lopez 1976); 2.7 days at 20°C for *L. hospitalis* (Voth 1972) and 1.3-1.7 days at 23°C for *L. dissimulatus* (Lewis, 1973). Although it was not the intent of the present study to estimate egg maturation, nauplii were noted to appear in cultures between 1 and 3 days post collection at 13-14°C. Since ovigerous females are often seen with egg sacs at different states of maturation, as indicated by the level of pigmentation in the egg strings (Roth 1988), it may not be valid to make the assumption that age distribution is uniform among a group of females (Johnson & Albright 1991a) thus explaining the discrepancies in developmental times cited above.

In general, the duration of the first nauplius stages is much shorter than the second nauplius stages in nearly all caligid copepods which have been studied, ranging from 0.4 days at 15°C to 1.5 days at 9.2°C (Johannessen 1978; Johnson & Albright 1991a). In the present study, at 10.7°C, copepodids were first seen approximately 2-3 days after the appearance of naupliar stages in lice cultures. More accurate time to development for the copepodid stage at 5, 10 & 15°C have been determined to be 9.3, 3.6 and 1.9 days respectively (Johnson & Albright 1991a). At 11 and 19°C Johannessen (1978) calculated the developmental time to be 2.6 and 1.4 days respectively. At 12°C

Wootten *et al.* (1982) calculated the developmental time to be 1.9 days.

The development of the copepodid stages marks a major transition in the morphology and behaviour of the salmon louse. The copepodid is much more streamlined than the naupliar stages (Johnson & Albright 1991c). Following the moult to the copepodid, second antennae transform into large clasping hooks which the animal uses to attach to the host. Wootten *et al.* (1982) reported that free living copepodids remained active for up to 4 days at 12°C. A longer time of 6 days was reported by Voth (1972) for *L. hospitalis* at 15°C. At a higher temperature (23°C) Lewis (1963) maintained copepodids of *L. dissimulatus* on artificial substrates for up to 3 days. It was suggested by Wootten *et al.* (1982) that, in the absence of a suitable food source (i.e. host), copepodids do not have the necessary energy stores to remain active for long periods; however, Johannessen (1978) noted that one individual copepodid remained alive for 30 days. In the present study, the duration of the copepodid stage was found to be 9-11 days which corresponds well with the time of 10 days given by Johnson and Albright (1991a). Since the main objective of the copepodid is host location, it is not surprising the stage is relatively long lived and was found to persist for up to 11 days. The relatively long developmental time required by copepodids may reflect a requirement to restore energy reserves once attached to the fish host (assuming feeding is initiated), or for the complicated process of producing a frontal filament for attachment.

The development of the four chalimus stages is less well documented. At 20°C, for *L. dissimulatus*, Lewis (1963) first noted the development of the chalimus stage after 3 days. At a somewhat colder temperature of 15°C, for *L. hospitalis*, Voth (1972) noted that the first chalimus stage appeared after 6-8 days. In the more recent study by

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Johnson & Albright (1991a) for *L. salmonis*, at 10°C, the first chalimus stage was reported to appear after 19 days. In the present study, development to the first chalimus stage was first seen after 10 days reflecting considerably faster developmental times despite a marginally higher temperature regimen. Subsequent developmental times for the appearance of more advanced developmental stages were also reduced. Thus the first adult female in the present study was obtained after 28 days whereas Johnson and Albright (1991a) report a development time of 52 days. However, in contrast, the duration of each of the developmental stages was in general agreement with that observed by Johnson and Albright (1991a) who found the duration of the various stages to be (in days): 5 for chalimus 1; 5 for chalimus 2; 9 for chalimus 3; 6 for chalimus 4; 8 for pre-adult 1 males; 10 for pre-adult 1 females; 9 for pre-adult 2 males and 10 for pre-adult 2 females. In the previously mentioned studies by Lewis (1963) and Voth (1972) a general trend of decreased developmental time with increase in developmental stage was noted. Apart from the copepodid stage, an increase in developmental time with developmental stage (excluding adults) was noted by Johnson and Albright (1991a). In the present study, developmental time for all the stages, excluding the free living naupliar stages and the adults, was found to be relatively constant. Due to the termination of the trial on Day 53 (from infection), the potential life span of the adults could not be calculated, although, in the presence of a host, the duration of the adults is believed to be in excess of 30 days as observed in the present study.

Most if not all the parasites studied were found attached to the body surfaces and fins, as reported by Wootten *et al.* (1982). In contrast, Johnson and Albright (1991a) reported that most of the copepods observed were found in the gill cavity. The latter

observations appear to contrast with the large body of data on sea lice, with little or no reference to gill attachment by *L. salmonis* chalimus. Neilson *et al.* (1987) reported that juvenile *Caligus* (species not identified but assumed to be *C. elongatus*) preferred to attach to the insertion of the fins in juvenile cod and to preopercular, interopercular, sub-orbital and the ventral half of the opercular bones in juvenile haddock. Taylor (1987) reported, that following laboratory infections of copepodids which settled onto the fish host, 80% were found on ventral body surfaces (opposed to dorsal surfaces) with 22 of 29 being found attached to the ventral fins. Similarly, Bron *et al.* (1991) found that, under conditions of laboratory infections, copepodids preferred fins and ventral surfaces. However, copepodids were also seen, to a lesser extent, in the gill cavity attached to the primary gill filaments (Bron *et al.* 1991). It is possible that the branchial attachment observed by Johnson and Albright (1991a) and Bron *et al.* (1991) may have been an artifact of the experimental infection conditions and subsequently contributed to the variation observed in the delayed and variable developmental times reported by the former.

Although adult females were first seen 28 days after the beginning of the experiment, the subsequent development of mature egg strings took approximately the same time. Unfortunately, second generation laboratory reared nauplii could not be obtained, thus allowing the completion of one generation in the laboratory. However, given that eggs from such females could hatch in approximately 1-3 days, the generation time (egg to egg) at $10.7 \pm 1.5°C$ is estimated to be 7.7 - 8 weeks. A similar estimate (7.5 - 8 weeks at 10 °C) was proposed by Johnson and Albright (1991a) under similar laboratory conditions. From observation of populations of lice at fish farm grow out sites, Wootten *et al.* (1982) estimated a generation time of 6 weeks at 9-12°C. Tully

(1989) using similar epidemiological techniques estimated the generation time for *L. salmonis* to be between 7 and 13 weeks depending on temperature.

Generation time is an important epidemiological parameter with respect to clinical control of lice at farm sites. A generation time of 8 weeks, as suggested by the present data, would indicate that if all stages of the life cycle could be killed by treatment (chemotherapeutic), treatment frequency would be significantly reduced. However, evidence from farm sites suggest that treatments may occur as often as every two weeks (Anon. 1992) suggesting that treatments are ineffective against a large reservoir of parasites infecting fish, or that recruitment rates are extremely high.

The use of fallowing has been suggested (Anon. 1992) as an alternative to breaking the life cycle of the louse. This is achieved by removing the stock from a given area (in some cases an entire loch system) and delaying restocking causing the resident lice population to crash due to a lack of available hosts. Such management polices would be dependant on the generation time and life span of lice. The present data suggests that although a period of eight weeks is required for one generation it does not account for the potential duration of the adult life span which may extend beyond the generation time. It is interesting to note that following an eight week fallow period in one Scottish Loch (Sunart) treatments with dichlorvos for sea lice were reduced from every two weeks to approximately once every three months or less (Anon. 1992). However, although the overall lice burden in the above mentioned loch was reduced, the ineffective nature of DDVP to the larval stages of lice would dictate that once lice populations begin to establish at a site, numbers would undoubtedly increase over time.

In light of these observations, it is perhaps surprising that there is little or no information on the toxicity of DDVP to larval stages of lice. In the present study, azamethiphos appeared to have little or no effect on larval survival following treatment, when administered either topically or intraperitoneally to infected fish. The lack of effect occurred even at a concentration of 1.0 mg/L (bath treatment) which is far in excess of the safe dose rate used to treat fish. The lack of response following IP injection is not surprising given the fast metabolic clearing times of compounds such as DDVP (Horsberg et al. 1990). In fact, unpublished work on the depuration of azamethiphos from salmon tissues indicates that azamethiphos is metabolised by fish quicker than DDVP (P. Dobson pers. comm.).

These findings are consistent with other studies which have shown that when organophosphorus compounds, such as DDVP and trichlorfon, are used to treat salmon infected with lice, repeat treatments are necessary due to a lack of effect on chalimus stages (Brandal & Egidius 1979; Rae 1979; Wootten et al. 1982). The mechanism for this apparent lack of effect is unclear as all stages of lice possess cholinergic nervous activity (Walday & Fonnum 1989b). It is therefore possible that the lack of AChE inhibition relates more to uptake than to lack of target sites for organophosphorus pesticides.

Resmethrin proved to have some degree of effect on larval lice, but the effects appeared to be subtle and required higher dose rates than would be clinically acceptable as indicated by toxic effects to treated fish. Following bath treatment, there were no observed mortalities 1 day post treatment, but there was a marked increase in the number of early chalimus stages (copepodids/chalimus 1 & 2) compared to late

chalimus stages (chalimus 3 & 4) which was most pronounced at 1.0 mg/L (MS) and in all the EC concentrations tested. This may suggest that pyrethroids show some degree of selective toxicity to larval sea lice and/or that the addition of an emulsifying agent enhanced the toxicity of the compound (Stammer 1959). However, these effects appeared short lived as lice in all groups appeared to recover 5 days from treatment. Such an effect is not unexpected given the characteristic "knock down effect" observed with many pyrethroids due to their action on peripheral nerves (WHO 1989a). In earlier trial work with an oil based pyrethrum (25%) formulation, Jakobsen & Holm (1989) noted a significant reduction in chalimus stages following topical treatment of lice infected salmon. Unfortunately, the authors did not verify the findings by observing dead lice and the decrease may be due to factors such as development.

Despite the high dose rates required, resmethrin was found to affect lice following IP injection to infected fish. Results from studies on related compounds such as permethrin have shown that pyrethroids are less toxic to fish intraperitoneally than topically (Glickman et al. 1981). The finding is of particular clinical interest and supports the earlier findings (Chapter 4) that pyrethroids in general may be suitable as oral chemotherapeutants.

The most pronounced effects were seen with the avermectin ivermectin, and the milbemycin, SKB7. Both compounds are marcrocyclic lactones, similar in structure and mode of action, the principle differences being the absence of a disaccharide chain at carbon 13 in the milbemycins (Fisher 1985). As discussed earlier, the mode of action of these compounds is to interfere with GABA mediated chloride ion channels. In bath treatments both compounds were highly effective in killing chalimus lice at dose

rates comparable to that which would be required to kill pre and adult lice as demonstrated previously for SKB7. Although the lowest dose rate tested for the topical application of SKB7 was 0.01 mg/L, the compound may be effective at lower dose rates, compared to the dose rate of 0.001 mg/L in the ivermectin trial, as milbemycins have been shown, in some instances, to be more toxic than the avermectins (Deecher, Brezner & Tanenbaum 1989). Interestingly, both compounds also produced sublethal effects, as indicated by inhibited moulting. Similar observations were made by Deecher, Brezner and Tanenbaum (1990) who reported that gypsy moth larvae (*Lymantria dispar*) exhibited weight loss, locomotor disruption and delayed moulting when exposed to sublethal concentrations of abamectin and "milbemycin D". Deecher *et al.* (1989) reported that the paralytic effects of milbemycin D were irreversible, but not for abamectin. It is unknown whether the sub-lethal effects of either compound (ivermectin, SKB7) are irreversible in sea lice chalimus stages; however, this is unlikely in light of the long recovery times used in the present study.

The compounds did differ in their effects on the lice when administered to fish intraperitoneally with SKB7 being the more toxic of the two and supports the findings of Deecher *et al.* (1990). The effects of ivermectin on the chalimus stage were documented by Palmer *et al.* (1987) who noted that oral treatment (0.2 mg/kg body weight/day) appeared to impair the development of chalimus stages. However, this dose rate was also found to produce undesirable side effects and has been noted to be toxic to fish in some instances (O'Halloran *et al.* 1992). Further work by O'Halloran *et al.* (1992) showed that a bi-weekly dose rate of 0.05 mg/kg body weight/day could be effectively used for the treatment of *Ergasilus labracis* infestations of salmon parr. Interestingly, no effect on lice was observed at a dose rate of 0.05 mg/kg in the present

study, suggesting that, at low dose rates, repetitive treatments are necessary. However, it may also reflect differences in the uptake and subsequent metabolism by fish as a result of the two different treatment regimens.

With respect to sea lice control, oral administration of ivermectin may present several problems. Høy et al. (1990) found that when ^{3}H-ivermectin was administered orally to salmon via gavage the compound was slowly absorbed by the gut with maximum concentration being reached in tissues after four days. Furthermore, of the total amount of label administered, only 29% was present in all tissues studied at day 4 suggesting that a significant proportion of the administered dose was either regurgitated or passed through the gut, with a large proportion of the excreted compound remaining unchanged.

The use of ivermectin or compounds such as SKB7 must be approached with caution due to narrow therapeutic margins (as stated earlier). Following topical administrations, both compounds (ivermectin & SKB7) were found to have therapeutic ratios of 10 with respect to acute toxicity. However, it is possible that SKB7 may show good efficacy at dose rates below 0.01 mg/L (bath treatment). Interestingly, both compounds required relatively high dose rates when used IP, with SKB7 being the more efficacious of the two. Although the toxicity of either compound was not investigated, it is interesting to note that an oral dose rate of 0.4 mg/kg of ivermectin was toxic to salmon (Palmer et al. 1987). It is possible that variable feeding responses in the treated fish led to individual overdose, which has been observed with trichlorfon (Brandal & Egidius 1977). Alternatively, based on the findings of Høy et al. (1990), poor uptake rates associated with oral ingestion cannot be compared to rates achieved

via injection. Toxicity has been observed in other fishes as well, including mottled sculpin (*Cottus bairdi*) and eels (*Anguilla anguilla*) at comparable dose rates (Heckman 1985; Tarachewski *et al.* 1988).

In salmon, ivermectin is sequestered in fatty tissues where it is slowly released (Høy *et al.* 1990). Following their studies, Høy *et al.* (1990) concluded that the blood brain barrier is poorly developed for salmon, accounting for the high levels of labelled compound observed in brain tissue. This may also account for the high toxicity observed in the present study. However, it does not explain the higher toxicity observed following uptake via the gills (topical treatments) over IP, a trend observed in all the compounds tested. The difference in the toxicity of the two administration regimens supports general findings that toxic compounds/pesticides are more rapidly taken up by the gills than they are orally (Jarvinen, Hoffman & Thorslund 1977).

Differences with respect to the toxicity of organic molecules in relation to target animal biology may be explained using the three component model suggested by O'Brien (1962) which states that the selective action of a given toxicant depends on (1) penetration selectivity, (2) target selectivity and (3) metabolic selectivity. The first of O'Brien's (1962) selectivity components relating to the structure of the cuticle is probably the most significant factor with respect to uptake (Hassal 1969).

The strong dichotomy observed between the toxicity of compounds such as resmethrin and azamethiphos to larval and adult/preadult lice presents a situation not unlike that observed for insects where susceptibility is influenced by the developmental stage, but the most tolerant developmental stage is different from species to species (Hole, Bell,

Mills & Goodship 1976). In contrast, decapod crustaceans, which show major similarities in cuticle structure and composition to caligid copepods (Kannupandi 1976; Stevenson 1985), larvae have been found to be more susceptible than adults to the acute toxicity of organochlorines (Armstrong, Buchanan, Mallon, Caldwell & Milleman 1976) and carbamates (Buchanan & Stewart 1970).

Limited available information suggests that the caligid cuticle, in particular *Caligus savala*, is very similar in design and composition to decapod crustaceans (Kannupandi 1976). However, Kannupandi (1976) also found that the structure of the cuticle varied somewhat from region to region, particularly with respect to relative thickness and the degree of hardening. Furthermore, it was also shown that in the allied pennellid copepod, *Pennella elegans*, in which the head and a portion of the neck lie in intimate contact with the fish host, the cuticle of the head was particularly porous and lacked an epicuticle (Kannupandi 1976). It is not known whether the structure of the cuticle in caligid copepods changes with successive development and it is possible that, should such differences exist, differences in pesticide penetrability might occur. Difference in uptake rate have been shown to account for differences in susceptibility to pesticides between different developmental stages in insects (Ramakrishnan, Chintalwar & Banerji 1989). Furthermore, given the differences in the general morphology between nauplius, copepodid, chalimus and post chalimus stages, there is no information on the relative surface area to volume ratio of various developmental stages of caligid copepods and it may be theorized that the relatively small surface area of early developmental stages offers less area for passive and/or active pesticide uptake.

The second of O'Brien's postulates refers to target specificity. With respect to OPs it

is known that all stages of lice (immature eggs excepted), contain relatively similar amounts of the target enzyme acetylcholinesterase (Walday & Fonnum 1989b). However, it should be noted that OPs can be toxic to insect eggs before nervous differentiation takes place (Hole *et al.* 1976). Thus one cannot ascribe differences in, or lack of, target sites to explain the apparent lack of toxicity in larval stages of lice. However, it might be speculated that although target sites/systems are present, they are not fully functional. Although the nature of the specific target sites for pyrethroids is not fully understood (WHO 1989a), it is likely that there is no appreciable difference in the availability of target sites. This is supported by the finding that ivermectin and SKB7 were found to be highly toxic to all stages of lice. Thus it can be concluded that target site specificity may not account for the discrepancies observed with respect to the toxicity of OPs and, to a lesser extent, pyrethroids to larval lice.

The results would suggest that, while some compounds show preferential toxicity to adult lice, others do not. Such a discrepancy is obviously mediated by two factors, the first relating to the chemical structure of the molecules in question, and the other to differences in the structure/metabolism of the lice. The last of O'Brien's (1962) postulates, pesticide metabolism, might offer an alternative explanation for the differences observed in compound toxicity and relates both to chemical structure and possible metabolic processes.

The rate at which organic molecules enter biological tissues depends primarily on their solubility in lipids, most often measured as the octanol/water partition coefficient (Kow) (Neely, Branson & Blau 1974). Furthermore there is a strong correlation between Kow and the aqueous solubility of organic chemicals (Chiou, Freed,

Schmedding & Kohner 1977). Thus the more insoluble a compound, the higher the Kow. In the present study, the molecules which had the highest Kow appeared to be the most toxic to larval lice (Table 7.4).

Table 7.4. Octanol water partition coefficients (Kow) and aqueous solubilities (mg/L, 20°C) of dichlorvos, azamethiphos, resmethrin and ivermectin[1].

Compound	Kow	Solubility
Dichlorvos	29.5[2]	10.01
Azamethiphos	11.2[3]	1.1[3]
Resmethrin	2900[5]	< 1.0[4]
Ivermectin	9900[6]	0.008[7]

[1]Data not available for SKB7
[2]Who 1989b.
[3]P. Dobson pers. comm.
[4]Worthing & Walker 1987.
[5]WHO 1989a.
[6]Wislocki et al. 1989.
[7]May & Wasik 1978.

It is possible that the relative amount of lipid or wax esters may be different in various developmental stages of lice. Such differences have been observed in calanoid copepods where the percentage of wax present in the total body lipids increased with each successive copepodid stage, but was relatively constant or reduced in adult stages (Kattner & Krause 1987). Furthermore, various types of fatty acids and alcohols were found to either decrease or increase, depending on the acid or alcohol in question, during development (Kattner & Krause 1987). Increased levels of wax esters and lipids correspond to periods of active feeding and/or shortly after hatching (i.e. prior to yolk absorption) (Kattner & Krause 1987; Blades-Eckelbarger 1991). Thus it may follow that different relative levels of lipids or wax esters in different development stages act as a storage site for highly lipophilic compounds such as the avermectins and

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milbemycins, which due to their resistance to hydrolysis may accumulate to toxic levels.

Lastly, differences in metabolism in various stages of lice may account for varying degrees of ability to cope with absorbed toxicants. The PCB Aroclor 1254 was found to selectively alter haemolymph chloride regulation in juvenile grass shrimp, *Palaemonetes pugio*, resulting in high toxicity, the effect not being observed in adults (Roesijadi, Anderson, Petrocelli & Giam 1976). Although in the case of *Lepeophtheirus salmonis* higher toxicity was observed for adults when treated with azamethiphos and resmethrin, the results from Roesijadi *et al.* (1976) indicate that metabolic detoxification pathways in crustacea may exist in different developmental stages. Differential toxicity of phosphine to different developmental stages of insect pests has also shown to be related to differences in metabolism (Ramakrishnan *et al.* 1989; Chaudhry & Price 1990).

Thus the toxicity of pesticides to larval lice may be related to permeability, lipid content and or metabolism. Compartmentalized lipids may store or bioaccumulate lipophilic substances where, in the case of complex molecules such as resmethrin, ivermectin and SKB7, metabolism may be delayed. Conversely, compounds such as azamethiphos and dichlorvos, which are relatively hydrophilic, and rapidly hydrolysed in solution, would not be expected to accumulate in larval lice, assuming that such lipid stores exist. However, physiological parameters associated with cuticular composition may also play a role in pesticide uptake.

8 Preliminary Studies on the Uptake, Disposition and Metabolism of Azamethiphos in the Salmon Louse, *Lepeophtheirus salmonis*.

8.1 Introduction

The recent finding that certain populations of lice have developed resistance to DDVP (Jones *et al*. 1992) has severe implications in the future control of sea lice in salmon farming. The first case of resistance in fish crustacean ectoparasites was reported by Lahav *et al*. (1962) who reported that populations of *Argulus* infecting carp in Israel had developed resistance to the organochlorine compounds lindane and endrin. Perhaps the most significant finding reported by these authors was that an 85 fold reduction in sensitivity to lindane was observed in parasites after only six treatments.

Previous work in this study has already shown that sea lice resistant to DDVP display reduced sensitivity to the related OP compound azamethiphos, which is currently being developed to replace DDVP. The lack of suitable alternative compounds has led to an intensive search for replacements as discussed earlier. Reports of resistance to candidate alternative sea lice compounds such as the pyrethroids (Keiding 1976; Sawicki, Farnham, Denholm & O'Dell 1981; Mekuria *et al*. 1991) and ivermectin (Van Wyk & Malan 1988; Echevarria & Trindade 1989; Craig & Miller 1990) is already widespread in insect and helminth populations respectively. In many cases, such as with fly or mosquito control, resistance has developed to as many as 5 or 6 different classes of pesticides (Sawicki 1975; Mekuria *et al*. 1991). Once selected, resistance mechanisms may persistence, in the absence of the selecting agent (i.e. pesticide), for as long as 20 years (Sawicki 1975).

Although more suitable non-chemical management techniques are desirable, chemotherapy will remain an important component of any integrated sea lice control strategy. Despite the inevitability of resistance, an understanding of the mechanisms involved can prolong the useful lifespan of a particular and subsequent chemotherapeutants (Sawicki 1979). In general, resistance is the result of one or more of the following mechanisms, 1) uptake, 2) detoxification and 3) target site sensitivity (Sawicki 1979). To date there has been little or no research into the physiological processes in caligids as they relate to chemotherapy. Thus, a series of preliminary studies were initiated to study, in general, uptake rates and distribution of azamethiphos in lice and the effects of detoxification modulators on a population of lice resistant to DDVP. In addition, data were obtained on the sensitivity of lice to pyrethroids which show different sensitivities to OPs.

Two compounds, piperonyl butoxide (PBO) and bis(*p*-nitrophenyl) phosphoric acid (BNPP) were chosen to identify possible detoxification pathways in lice. PBO, is a well known inhibitor of the mixed function oxygenases (MFO) and breaks down a wide variety of toxins in animal tissues (Neff 1985; Payne 1977), and is often combined with several insecticides, notably pyrethrins, to increase toxicity (Worthing & Walker 1987). BNPP binds to non-specific esterases which hydrolyse a wide variety of molecules, particularly OPs and pyrethroids (Sawicki 1979; Glickman *et al.* 1981; 1982).

8.2   Methods

8.2.1 Sea Lice

All experiments were carried out *in vitro*, using the salmon lice, *Lepeophtheirus salmonis*, collected from salmon farms on the west coast of Scotland as described

previously. The source of lice for each of the trials is given below. All lice collected were maintained in a constant temperature room (12°C) in containers filled with pre-cooled, filtered sea water. Water in the containers was mildly aerated and changed daily to maintain adequate quality.

8.2.2 [¹⁴C]Azamethiphos

[2-^{14}C]pyridyl azamethiphos (*S*-6-chloro-2,3-dihydro-2-oxo1,3-oxazolo[4,5-*b*]pyridin-3-ylmethyl *O,O*-dimethyl phosphorothioate) (Figure 8.1) (IUPAC, Worthing & Walker 1987) was obtained as a 98% active ingredient (ai), technical crystalline solid with an activity of 1.57 MBq/mg (23.5 mg) from Ciba-Geigy Agrochemicals. The compound was subsequently dissolved in 50 mL ethyl alcohol (ETOH) (470 mg/L) which was then used as a concentrated stock for the bioassays. The stock was stored at 4°C in an opaque glass container.

8.2.3 Liquid Scintillation Counting

8.2.3.1   Experiment 1 - Exposure Concentration

Lice were obtained from a salmon farm located in Loch Melfort (Kames Fisheries Ltd). Groups of 5 adult female lice were randomly placed into beakers with 400 mL of pre-cooled, filtered sea water. Concentrations of 0.0 (control), 0.1, 0.2, 0.3, 0.4 and 0.5 mg/L were prepared by diluting the stock (470 mg/L) [^{14}C]azamethiphos solution with sea water. Solutions in the beakers were then changed with the appropriate test solution and allowed to stand static for 1 hour.

Following the 1 hour exposure, 3x 0.1 mL aliquots were taken from each of the beakers and blotted onto small pieces of filter paper (1.0 x 0.5 cm; Skatron AS, Receptor Binding-Filtermats) and allowed to dry. The solutions in each of the beakers were then discarded and each of the beakers rinsed 3x with 500 mL of fresh sea water (FSW).
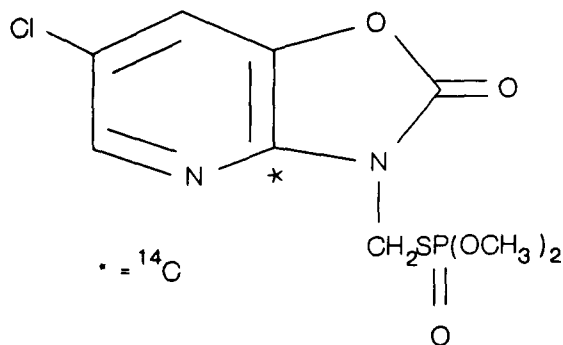

Figure 8.1. Diagram of [^{14}C]azamethiphos showing location of ^{14}C labelled carbon.

Following the three rinsings, 3 lice were removed from each beaker and placed into scintillation vials (20 mL, High Performance Glass, Canberra Packard) and left open to dry for 24 hours. 0.3 mL of tissue solubilizer (Soluene-350, Canberra Packard) was then added to each vial. The vials were then capped, vortexed, and allowed 24 hours to digest. Following the digest, 5.0 mL of scintillant (Ecoscint, Canberra Packard) was added to each of the vials and the vials lightly vortexed.

In addition to the lice samples, 3x 0.1 mL aliquots were taken from the 0.5 mg/L beaker following each rinsing and blotted onto filter paper as described above. The 0.5 mg/L beaker was then given three rinsings with 3 x 50 mL of ETOH. 3x 0.1 mL aliquots of ETOH were blotted onto filter paper following each rinsing. The three remaining lice were then placed into scintillation vials and processed as described above. Vials were then counted with a United Technologies Packard 2000CA Tri-Carb Liquid Scintillation Analyzer for 1 cycle of 5.0 min each.

In addition to the above test solutions, a series of concentration standards (0.5, 0.05, 0.005 & 0.0005 mg/L) were prepared by serially diluting the 0.5 mg/L [^{14}C]azamethiphos solution. Triplicate 0.1 mL aliquots of each dilution were blotted onto filter paper as well as onto individual lice which had been placed into scintillation vials. The vials were then allowed to dry and processed as described above.

8.2.3.2 Experiment 2 - Exposure Time

In addition to the above experiment, lice were also exposed to 0.5 mg/L [^{14}C] azamethiphos for various time intervals ranging from 10 min to 60 min. Each group consisted of 8 adult female *Lepeophtheirus salmonis*, except the 60 min exposure group

which contained 11. Separate test solutions were prepared by adding 535 μL to 500 mL sea water. Lice were exposed to test solutions by replacing the contents of the beakers holding the lice with prepared [^{14}C]azamethiphos test solutions. One control groups was used which contained 535 μL ETOH and was exposed for 60 min.

At the beginning of each exposure period, 3x 0.1 mL aliquots were removed from each of the beakers and blotted onto filter paper. At the end of each exposure, each beaker was rinsed 3x with 500 mL of FSW after which 3 lice were individually placed into scintillation vials. In addition, 5 lice from each group were mounted for autoradiography (see below). For the 60 min test group, 3 additional lice were left in the beaker and given three additional rinses with 50 mL ETOH. 3x 0.1 mL aliquots were blotted onto filter paper after each rinsing. The remaining lice were then placed into individual scintillation vials. All lice and filter papers were allowed to dry out overnight and then were processed as described above.

Counts per minute (CPM) were converted to disintegrations per minute (DPM) by multiplying the CPM values by a quench correction factor based on a quench correction curve for ^{14}C (see Appendix 1).

The amount of compound absorbed, per louse, was estimated by converting DPM values to mg [^{14}C]azamethiphos using the following conversion factor:

$1 \ \mu Ci = 2.2 \ 10^6$ DPM

Therefore assuming[11] that the activity of [^{14}C]azamethiphos $= 1.57$ MBq/mg and since 1 MBq $= 27.03 \ \mu Ci$, then

1 mg [^{14}C]azamethiphos $= 1.57$ MBq $= 42.43 \ \mu Ci = 93,361 \ 10^3$ DPM or

1 DPM $= 0.0107$ ng [^{14}C]azamethiphos (predicted)

where:

Ci $=$ Curies

Bq $=$ Becquerels

8.2.4 Whole Body Autoradiography

Lice from the above time series experiment were mounted between two glass slides, compressed and allowed to dry for several days. Once dried, lice were then mounted, one dorsally, one ventrally, onto a sheet of cardboard (18 x 24 cm) with the aid of paper glue.

In addition to the above, a set of concentration standards was prepared by blotting onto the cardboard adjacent to the lice, 100, 50 and 10 μL aliquots of the 0.5 mg/L stock [^{14}C]azamethiphos solution (representing 50, 25 and 5 ng (predicted) total [^{14}C]azamethiphos respectively).

A sheet of Amersham βmax hyperfilm was then placed over the lice, which was subsequently covered with another piece of cardboard. Both cardboard sheets and the film were then placed between glass plates (5.0 mm thickness) which were tightly

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[11] Relative activity was assayed by Ciba-Geigy prior to shipping.

bound together. The film was then placed into a light proof polythene bag for 1 week.

Following exposure the film was developed using the following schedule:

1 - Kodak D19 developer for 5 min

2 - H_2O rinse 2 min

3 - Kodak Hypam fixer for 2 min

4 - H_2O rinse 5 min

All work with negative film was carried out in a dark room, without the aid of a safelight. Once negatives were developed, printing was carried out under safelight illumination. Developed film was lightly coated with Kodak "photo flow", by placing 5 mL into the water bath and moving the film through the surface several times to prevent streak marks appeared on the film surface during drying. Prints were made with Kodak film, using an automated developer (Ilford 2150 R).

8.2.5 Metabolic Inhibition Studies

8.2.5.1 Sensitivity Trials

Two sites were selected which were believed to represent sensitive and resistant lice populations as estimated by the site managers' previous success with AQUAGARD SLT* treatments. Sites studied included Loch Spelve (resistant) and Loch Melfort (sensitive). Two compounds, resmethrin (20% MS) and azamethiphos (50% WP) (see Chapter 4 for compound supplier details), were used to test the relative sensitivity of the lice following 1 hour exposures *in vitro*. In addition, one trial was completed with DDVP (AQUAGARD*, 50% w/v DDVP) with lice from the Spelve site.

Lice were collected in the manner described previously. All exposures were 1 hour long at a temperature of 9.5 ± 0.5°C and salinity of 32.0 ± 2‰. Following exposure, each beaker was rinsed 2x with FSW and then refilled with fresh, pre-cooled, filtered FSW and given a 24 hour recovery/observation period. Response to the trial was recorded after 1 and 24 hours as described previously.

Each trial consisted of 1 replicate (per concentration) consisting of 10 lice per replicate. Concentrations tested are given below. Since all compounds were soluble in water at the concentrations test no solvent control was used. Trials were discarded and repeated where more than 10% of the lice in the sea water controls were found moribund/dead after 1 and/or 24 hours.

8.2.5.2   Inhibition/Toxicity Trials

Following confirmation of reduced sensitivity to DDVP in the Loch Spelve lice, the site was chosen as a source of lice for the metabolic inhibition studies. Compounds tested for toxicity were azamethiphos, resmethrin and DDVP (as described above). Metabolic inhibitors studied were the mixed function oxygenase inhibitor, piperonyl butoxide (PBO) and the esterase inhibitor, bis(p-nitrophenyl) phosphoric acid (BNPP).

PBO was obtained as a 90% ai technical liquid (Aldrich) which was solubilized in ETOH and then diluted in FSW as necessary. All three pesticides (DDVP, azamethiphos & resmethrin) were tested singly and in combination with 0.1 and 1.0 mg/L PBO at varying pesticide concentrations. Concentrations tested are given below. Sea water and ETOH controls were also tested.

BNPP was obtained as a 99%+ ai w/w sodium salt (BDH) which was solubilized in ETOH and then diluted in FSW as necessary. For these trials, azamethiphos and resmethrin were tested singly and in combination with 10.0 mg/L BNPP at varying pesticide concentrations. Concentrations tested are given below. Separate sea water and ETOH controls were also tested.

Protocols used were those described above for the sensitivity trials.

8.3 Results

8.3.1 Liquid Scintillation Counting

Counting efficiency achieved throughout the label studies was between 94-95%. There was an excellent correlation ($r^2 = 0.999$) between predicted DPM counts for the concentrations tested and measured values (100 μL samples) for the various concentrations tested (Figure 8.2; see also Table 8.1). No differences were observed between scintillation vials containing either lice or filter papers spiked with test solutions (Figure 8.3). The limit of the sensitivity of the system was found to lie between 5 and 10 ng [^{14}C]azamethiphos (Figure 8.3, Table 8.1).

It is difficult to ascertain what effect rinsing treated lice with ETOH may have had on the total amount of absorbed compound. Analysis of the washings (100 μL aliquots) showed no appreciable difference in activity (DPM) between washing samples (Figure 8.4), although there did appear to be slightly higher activity in washing following the first rinsing with ETOH (Figure 8.4). Activity measured from whole lice preparations also indicated that ETOH may have been extracting a certain amount of labelled compound from lice (Figure 8.5). There was a 58% reduction in the amount of

detectable label in lice following 3 rinsings with ETOH in one trial (given as Experiment 1 in Figure 8.5). However, following a second trial (given as Experiment 2 in Figure 8.5) the reduction was only 11.0% and was found not be significant ($t = 0.495$, $P = 0.067$).

Table 8.1. Total activity, DPM ± SD in 0.1 mL test solution samples of filter paper spiked with [^{14}C]azamethiphos (corrected for background activity).

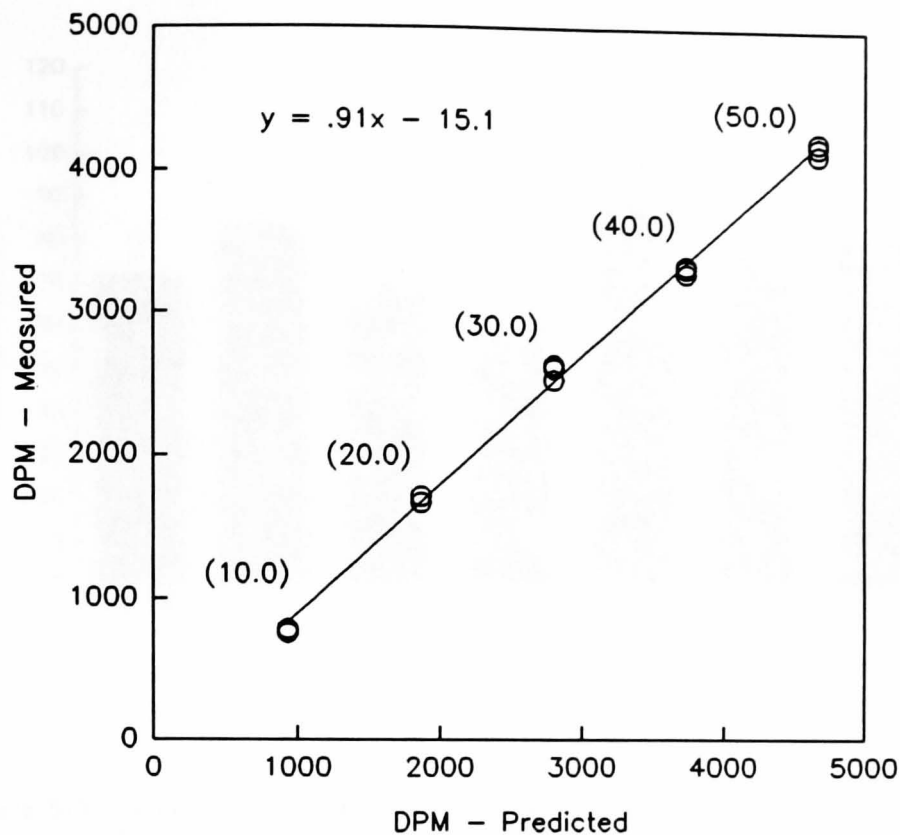| Est. amount of [^{14}C]azamethiphos in sample (ng)	Trial - Exposure period (min)	DPM Count Measured	DPM Count Predicted
10.0	1 - 60	764.61 ± 15.77	933.61
20.0	1 - 60	1701.80 ± 25.14	1867.22
30.0	1 - 60	2595.60 ± 54.67	2800.83
40.0	1 - 60	3320.17 ± 27.24	3734.44
50.0	1 - 60	4183.20 ± 43.83	4668.05
50.0	2 - 10	4123.49 ± 55.73	
50.0	2 - 20	4212.06 ± 85.79	
50.0	2 - 30	4312.00 ± 62.98	
50.0	2 - 40	4273.22 ± 39.79	
50.0	2 - 50	4238.78 ± 49.96	
50.0	2 - 60	4188.38 ± 73.48	

Figure 8.2. Linear regression for predicted and measured DPM (disintegrations per min) counts for several quantities of [^{14}C]azamethiphos (() = quantity in ng).
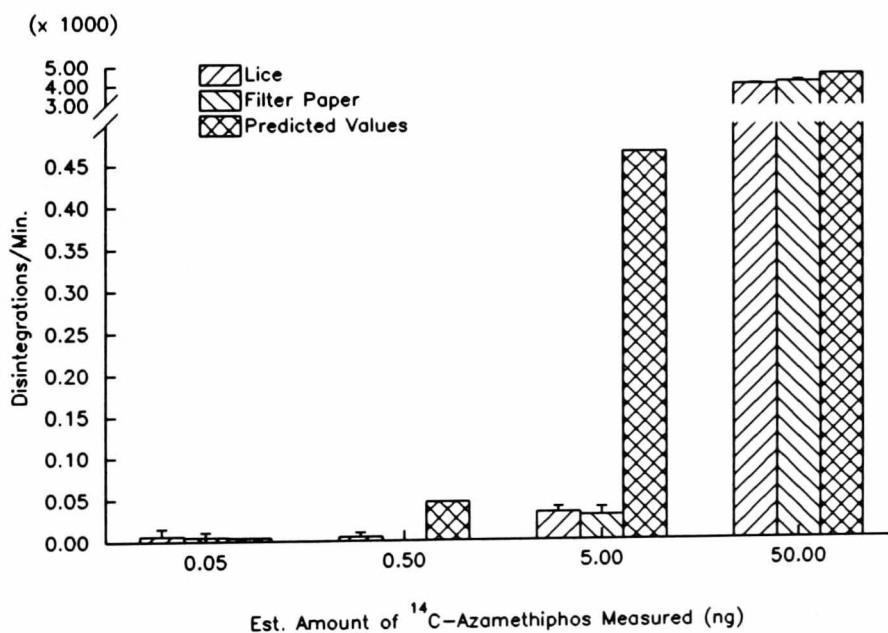

Figure 8.3. DPM counts, measured and predicted, for filter paper and lice samples spiked with various amounts (predicted) of [^{14}C]azamethiphos (bars = SD).
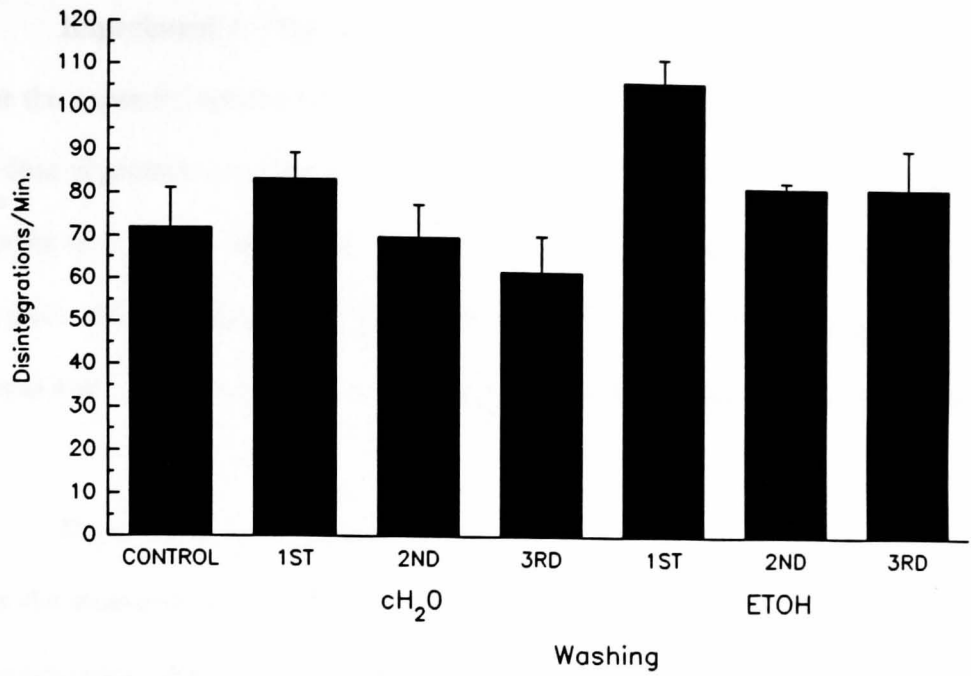
Figure 8.4. DPM counts recorded for washing solutions from sea lice following exposure to [^{14}C]azamethiphos (control = H_2O, bars = SD).
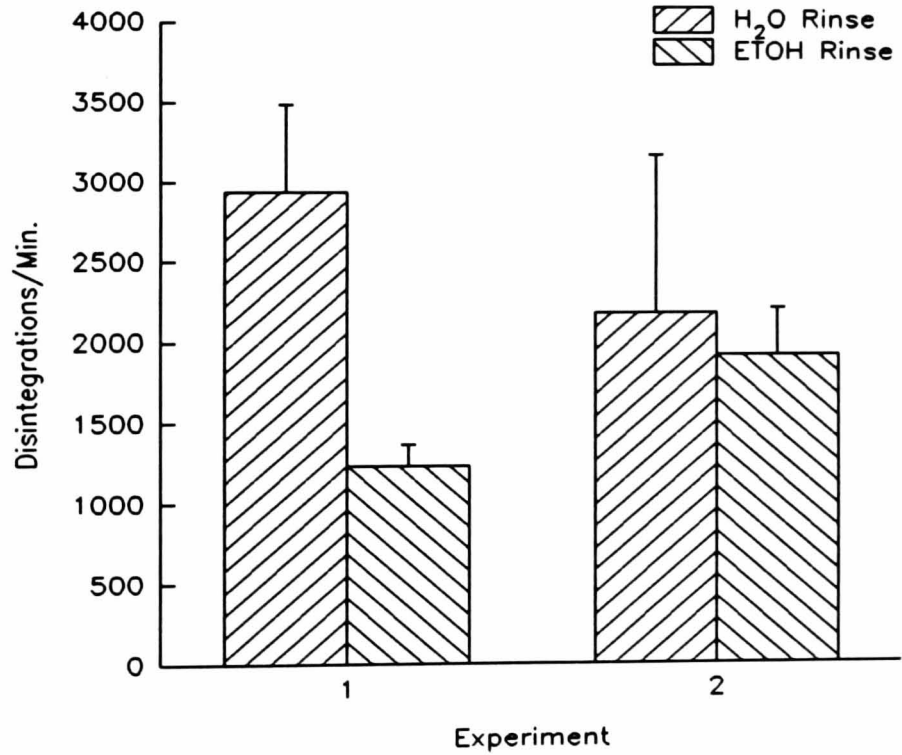

Figure 8.5. Comparison of DPM counts recorded for sea lice exposed to [^{14}C]azamethiphos following washing with cH_2O and ETOH (bars = SD).

8.3.1.1   Experiment 1 - Exposure Concentration

Results for the exposure concentration trial are summarized in Figure 8.6. Lice showed a dose dependant increase in the total amount of absorbed label up to exposure concentrations of 0.4 mg/L with an estimated 41 ng of compound being absorbed by individual lice. At the higher concentration of 0.5 mg/L, the amount of compound absorbed was markedly reduced (21 ng), possibly due to toxic effects of the compound.

8.3.1.2   Experiment 2 - Exposure Time

Results for the exposure time experiment are summarized in Figure 8.7. As with the previous experiment, uptake of [^{14}C]azamethiphos appeared to be time dependant for the first 50 min of the exposure. The amount of compound absorbed over the next ten minutes appeared to stabilize with no further increases observed. The highest amount of label absorbed was estimated to be 29 ng of compound/louse (40 & 50 min - Figure 8.7).
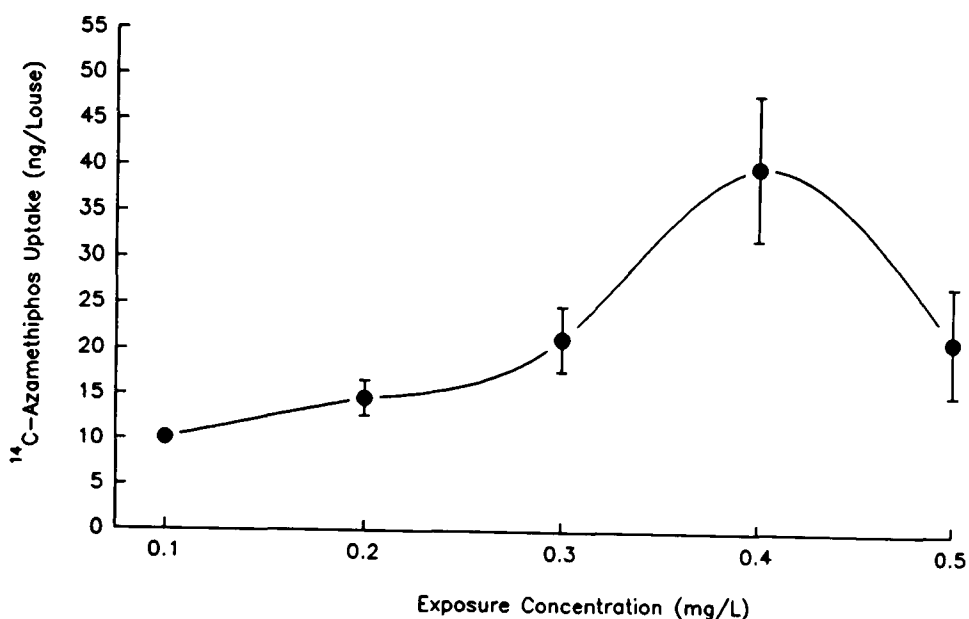
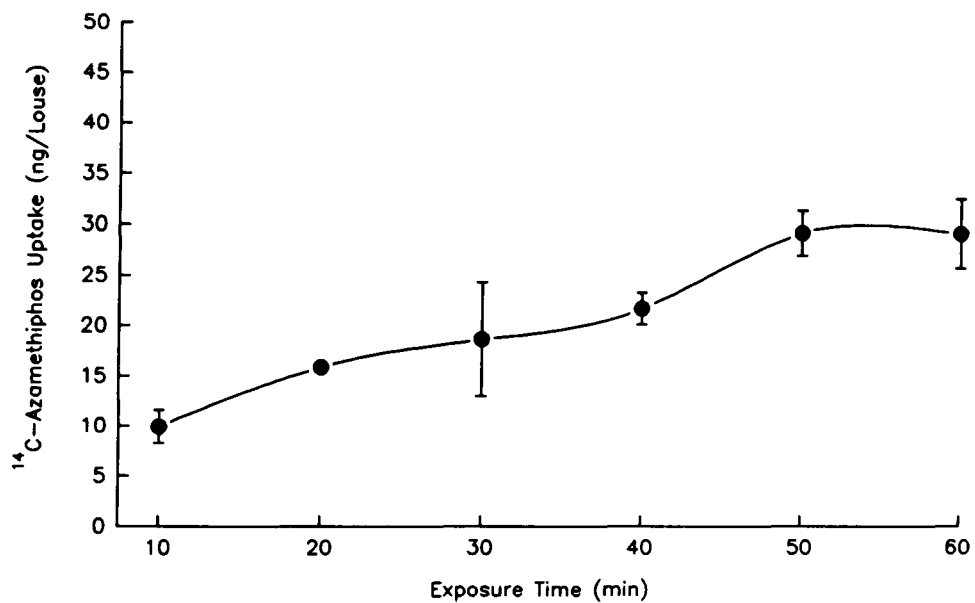**Figure 8.6.** Uptake of [^{14}C]azamethiphos in sea lice following 1 hour exposure *in vitro* (bars = SE).

Figure 8.7. Uptake of [^{14}C]azamethiphos in sea lice following exposure to 0.5 mg/L for 1 hour *in vitro* (bars = SE).

8.3.2 Whole Body Autoradiography

Results for the whole-body autoradiography are given in Plates 1 & 2 (for reference to anatomical nomenclature see Figure 2.2). Overall no defined "routes" of absorption were evident with label being primarily associated with the frontal plates, 1st antenna and the hind gut (Pates 1 & 2). Following ten minutes exposure, label appeared to be associated with the frontal plates and first antenna, with small amounts of label being associated with the oviducts and anus in one individual. Following a further 10 minutes, radioactivity appeared to be present in the hind gut of all individuals. By 40 minutes exposure, the compound appear to spread throughout the cephalothorax, with higher concentrations being detected around the peripheral margin and along the lateral sutures. By 60 minutes exposure, labelled material appeared to be generally spread throughout individuals, with highest concentrations being associated the second antennae and the anus. Labelled material was also found to be associated with certain appendages, notably the maxillipeds and the second and third thoracopods, with increases in concentrations over time.

Plate 8.1a. Whole body autoradiograph of *Lepeophtheirus salmonis* (A ♀) exposed to [^{14}C]azamethiphos at 0.5 mg/L for varying time intervals (Group 1). ø, control; 10 - 60, 10 - 60 minutes exposure; D, dorsal; V, ventral; S1 - S3, standards (ng - predicted) (specimens are acutal size).
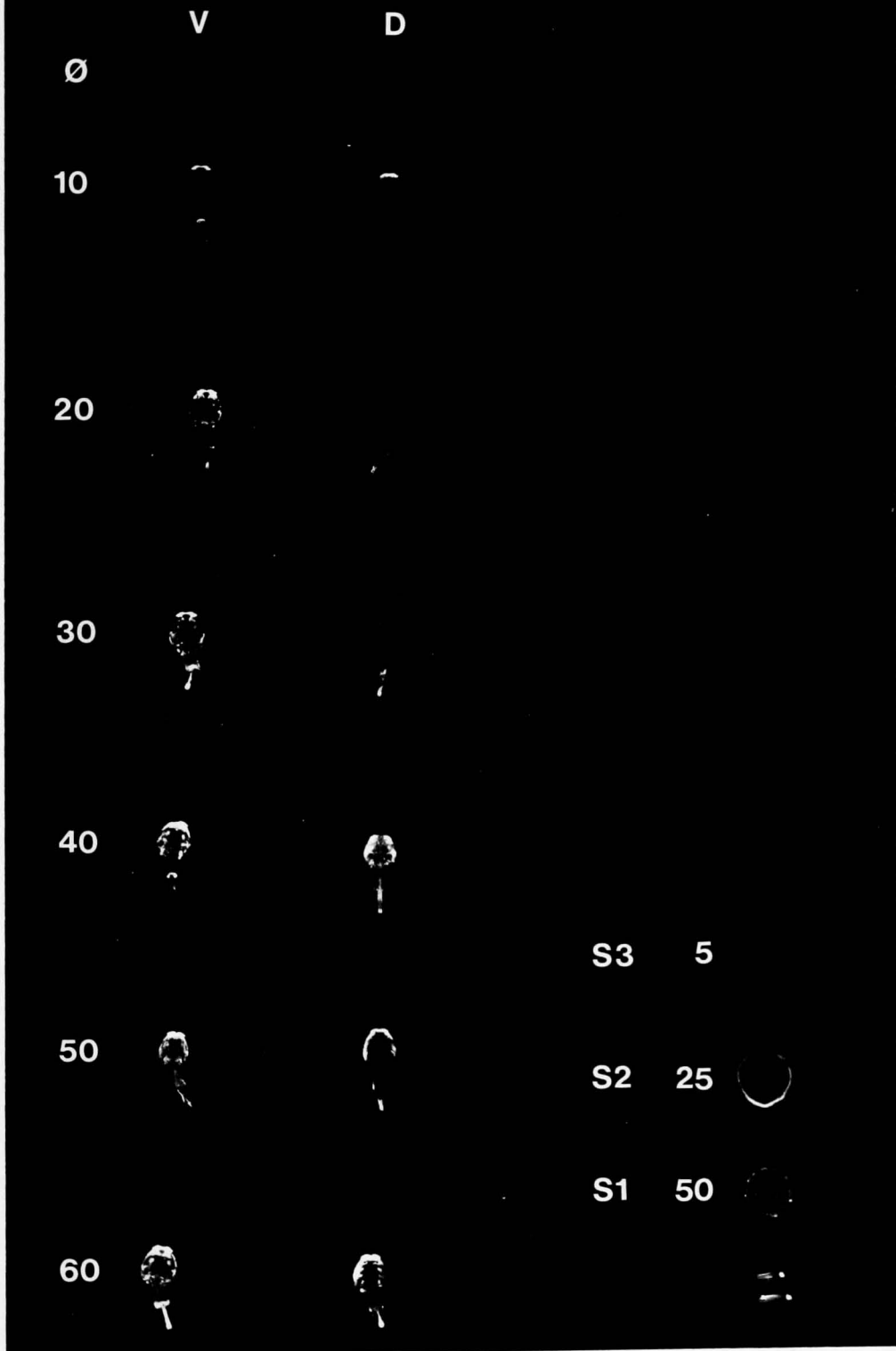
Plate 8.1b. Whole body autoradiograph of *Lepeophtheirus salmonis* (A ♀) exposed to [^{14}C]azamethiphos at 0.5 mg/L for varying time intervals (Group 2). ø, control; 10 - 60, 10 - 60 minutes exposure; D, dorsal; V, ventral; S1 - S3, standards (ng - predicted) (specimens are acutal size).

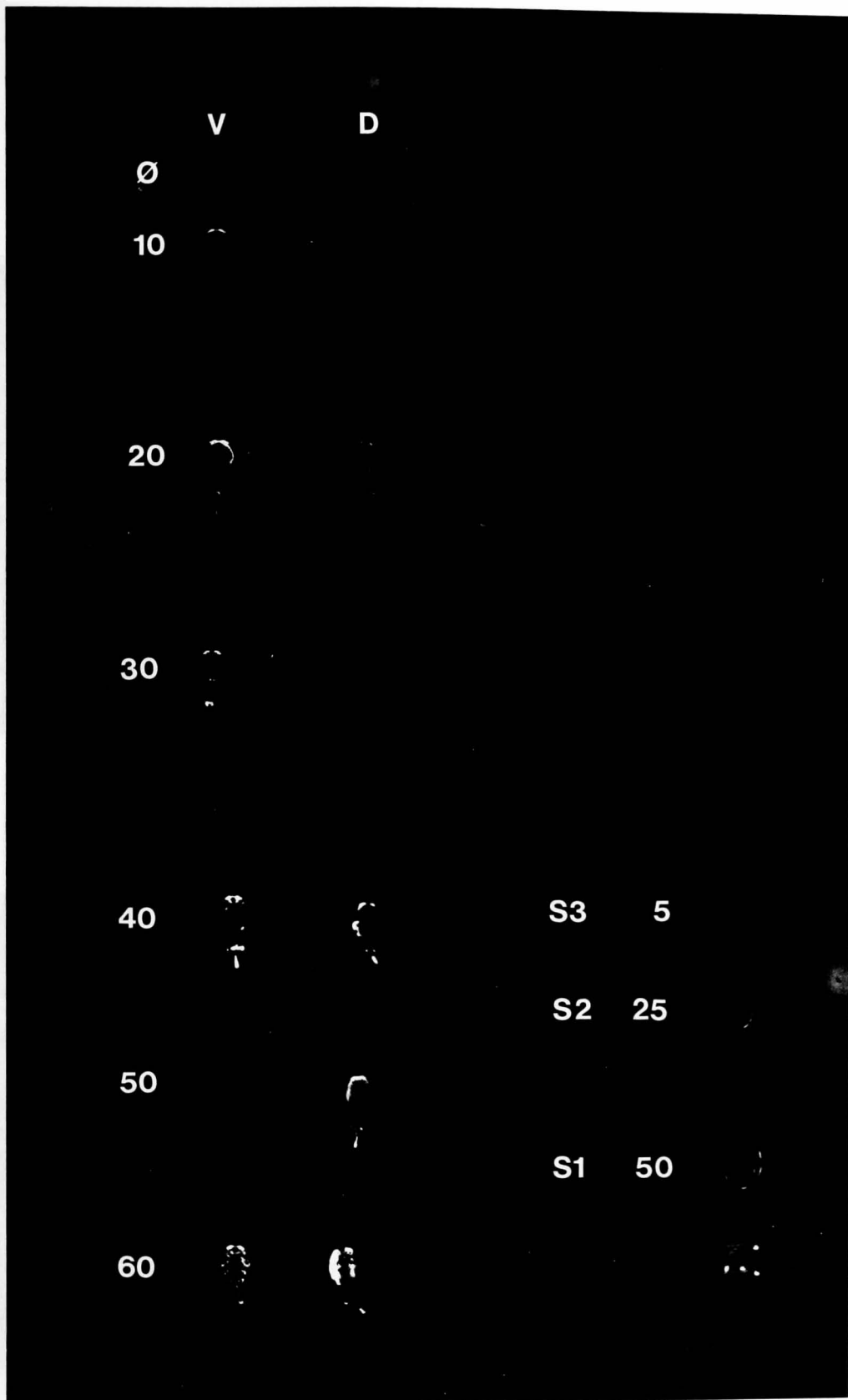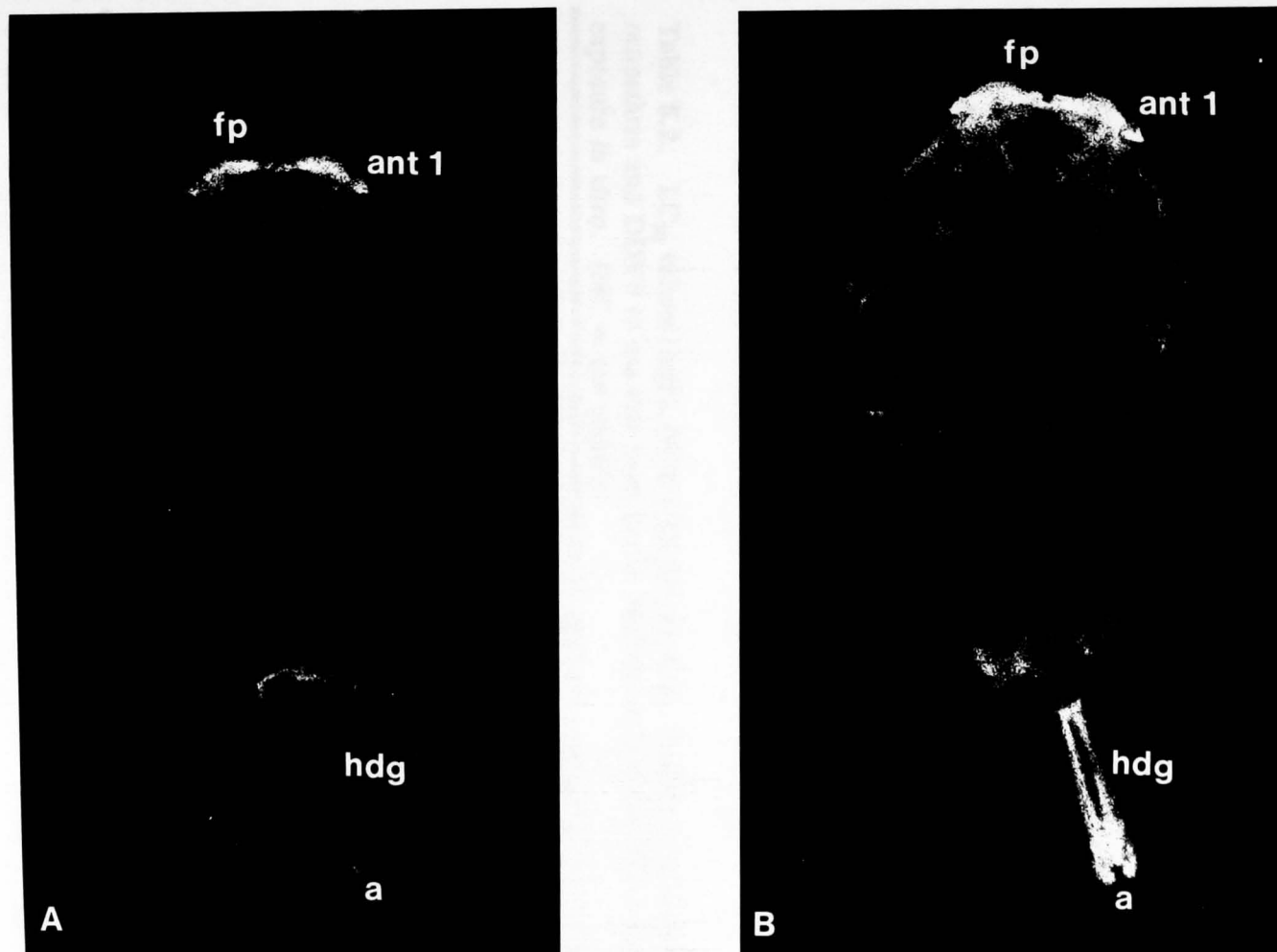**Plate 8.2.** Whole body autoradiograph of Lepeophtheirus salmonis (A ♀) following exposure to 0.5 mg/L [^{14}C]azamethiphos (dorsal view). A - 10 minutes exposures (mag. ≈ 8x). B - 60 minutes exposure (mag. ≈ 9x). fp, frontal plate; ant 1, first antenna; hdg, hind gut; a, anus.

8.3.3 Metabolic Inhibition Studies

8.3.3.1   Sensitivity Trials

Results for the sensitivity trials are summarized in Table 8.2. Lice from Loch Melfort were found to be more sensitive to azamethiphos than those from Loch Spelve. However, no conclusions could made with respect to the differential toxicity of resmethrin between the two populations. Overall, lice from both lochs were highly sensitive to resmethrin with 100% mortalities being recorded at concentrations of 0.0075 mg/L. In tests with both azamethiphos and resmethrin a delayed toxicity was observed, with higher mortalities being recorded after the 24 recovery period. Interestingly, there was little difference between the 1-h and 1+24-h results for the Loch Spelve lice exposed to DDVP.

Table 8.2. LC$_{50}$ values (mg/L (95% CI)) for the acute toxicity of azamethiphos, resmethrin and DDVP to sea lice from Lochs Melfort and Spelve following one 1 hour exposure *in vitro*. (NT = not tested).

Compound/ LC$_{50}$ Interval	Lice Source	
	Loch Melfort	Loch Spelve
Azamethiphos		
1-h	0.035 (.027-.045)	0.483 (.203-1.148)
1+24-h	« 0.0075	0.057 (.042-.078)
Resmethrin		
1-h	0.008 (.005-.014)	« 0.0075
1+24-h	0.003 (.002-.004)	« 0.0075
Dichlorvos		
1-h	NT	0.274 (.213-.353)
1+24-h	NT	0.315 (.235-.423)

8.3.3.2 Inhibition/Toxicity Trials

8.3.3.2.1 Piperonyl Butoxide

PBO appeared to have no synergistic effects when added to the test compounds (DDVP, azamethiphos, resmethrin) (Figures 8.9 - 8.11). The results obtained for individual test compounds, without PBO, confirmed earlier findings, with respect to toxic concentrations and delayed effects. Although delayed toxicity was observed with DDVP, the difference was marginal between 1-h and 1+24-h post exposure observations. Concentrations of DDVP in excess of 2.0 mg/L were required to produce 100% mortality in treated lice (24 hours after exposure), confirming the reduced sensitivity of the Spelve lice population (Figure 8.9).

As described above, delayed toxicity was observed for both azamethiphos and resmethrin. At a concentration of 0.25 mg/L azamethiphos, the mortality response ranged from 60 - 100% in the various treatment groups (Figure 8.10a). Concentrations in excess of 0.25 mg/L azamethiphos were required to produce a 100% mortality in all groups. Delayed toxicity was less evident in the lice treated with resmethrin, presumably to the overall higher toxicity of this compound. At a concentration of 0.025 mg/L resmethrin, the lice response was highly variable and ranged from 30 - 100% mortality. In general, the compound appeared more toxic in the absence of PBO, with high toxicity observed at concentration of 0.0075 mg/L (Figure 8.11b). PBO, in the absence of the pesticides was found to be non toxic to lice.

Figure 8.9. Acute toxicity of DDVP and DDVP + PBO combinations to sea lice following 1 hour exposures *in vitro* (response from beginning of exposure - A 1-h; B 1+24-h).

Figure 8.10. Acute toxicity of azamethiphos (AZA) and AZA + PBO combinations to sea lice following 1 hour exposures *in vitro* (response from beginning of exposure - A 1-h; B 1+24-h).
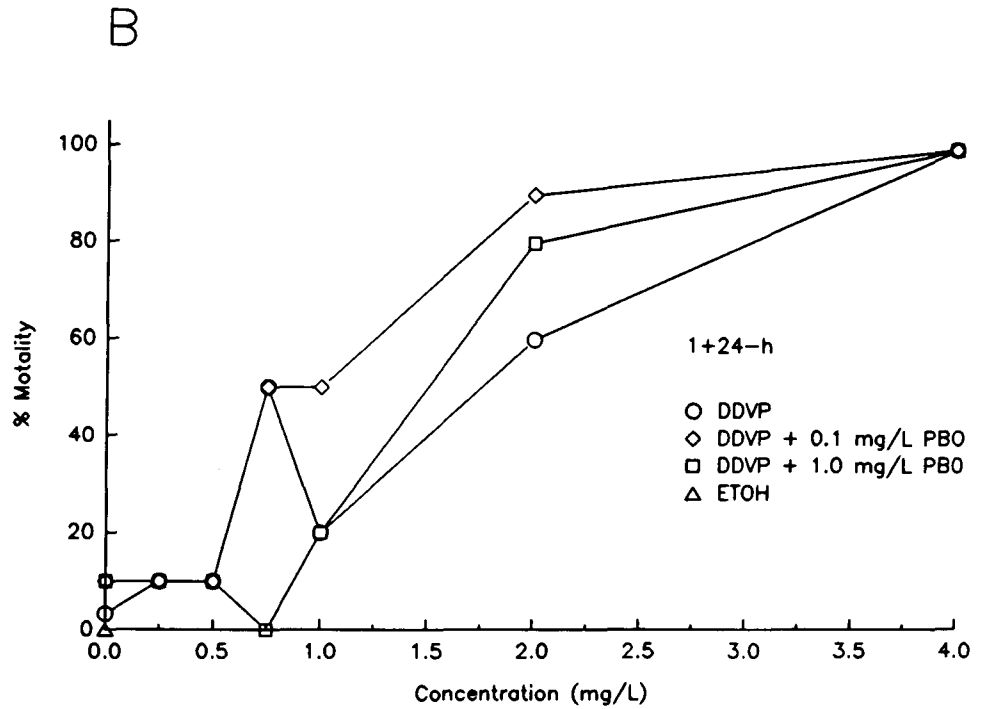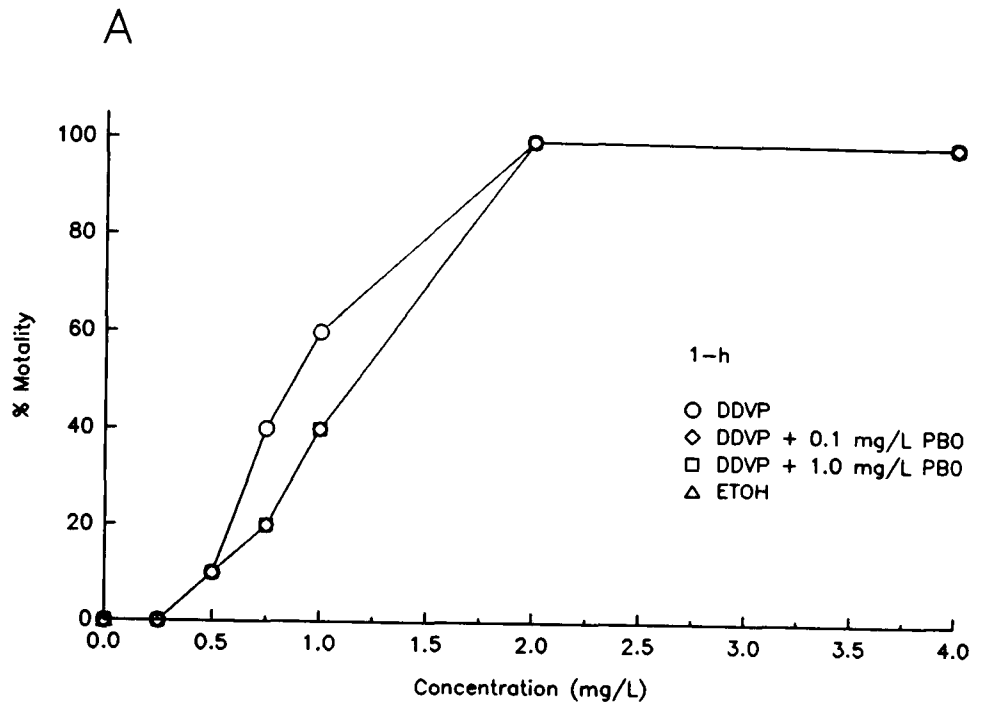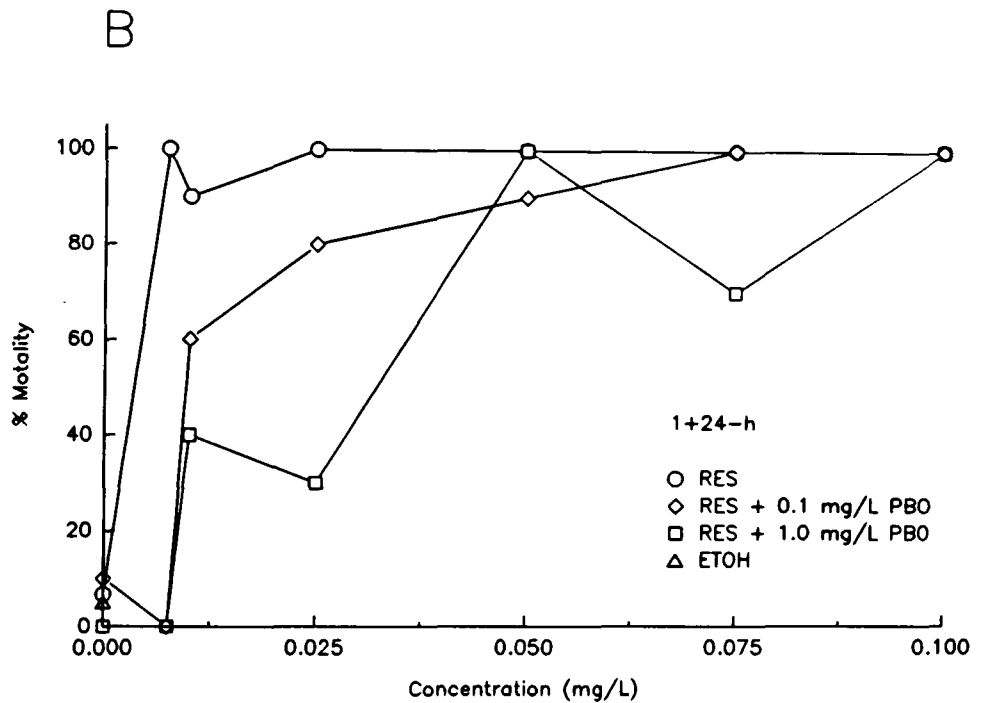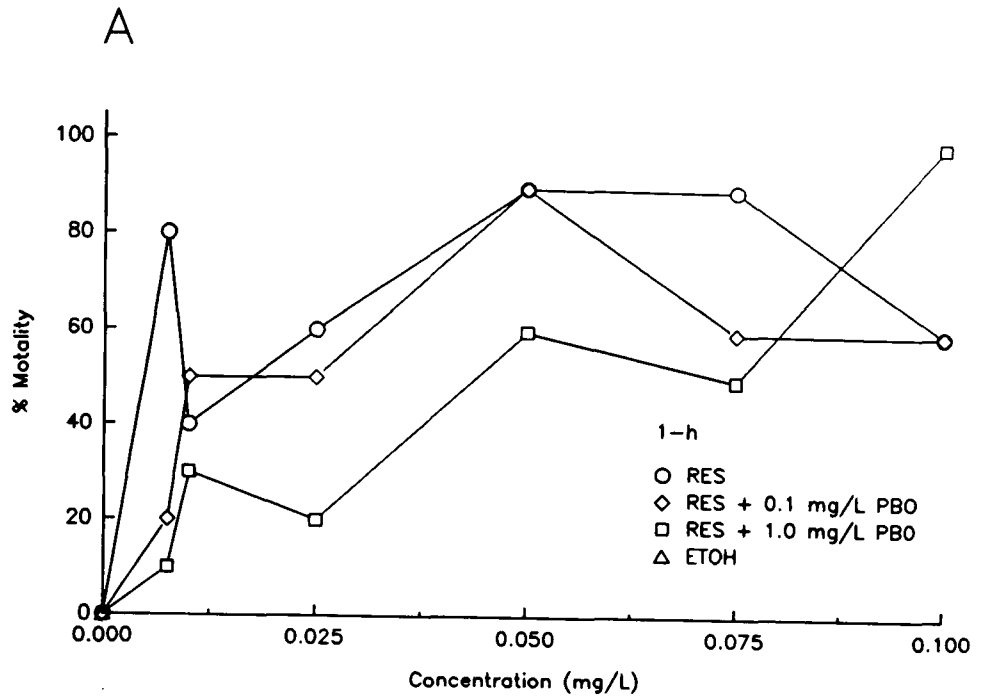
Figure 8.11. Acute toxicity of resmethrin (RES) and DDVP + PBO combinations to sea lice following 1 hour exposures *in vitro* (response from beginning of exposure - A 1-h; B 1+24-h).

8.3.3.2.2   bis(p-Nitrophenyl) Phosphoric Acid

In contrast to the results obtained with PBO, BNPP appeared to confer a synergistic

effect when combined with both azamethiphos and resmethrin (Figures 8.12 & 8.13).

However, it should be noted that only one concentration of BNPP was used (10.0

mg/L), which when tested in the absence of the pesticides was found to be somewhat

toxic to lice with a 30% lice response in BNPP-control groups after 1 hour followed by

a slight recovery (30%) during the 24 hour observation period.

As observed in previous trials, both azamethiphos and resmethrin were found to have a

delayed toxic effect towards lice. Lice showed a marked reduction in sensitivity to

azamethiphos (Figure 8.12), with 20% survival in lice treated at 0.5 mg/L. It should

be noted that in one ETOH control group, lice mortality was particularly high (60%).

However, given the lack of effect seen in all of the other trials, the result from this

particular replicate was not considered representative.

The synergistic effect of BNPP appeared to be more pronounced in trials with

resmethrin (Figure 8.13). Interestingly the toxicity of resmethrin, without the addition

of BNPP, appeared to be much less to lice than in previous trials (Figure 8.13), with

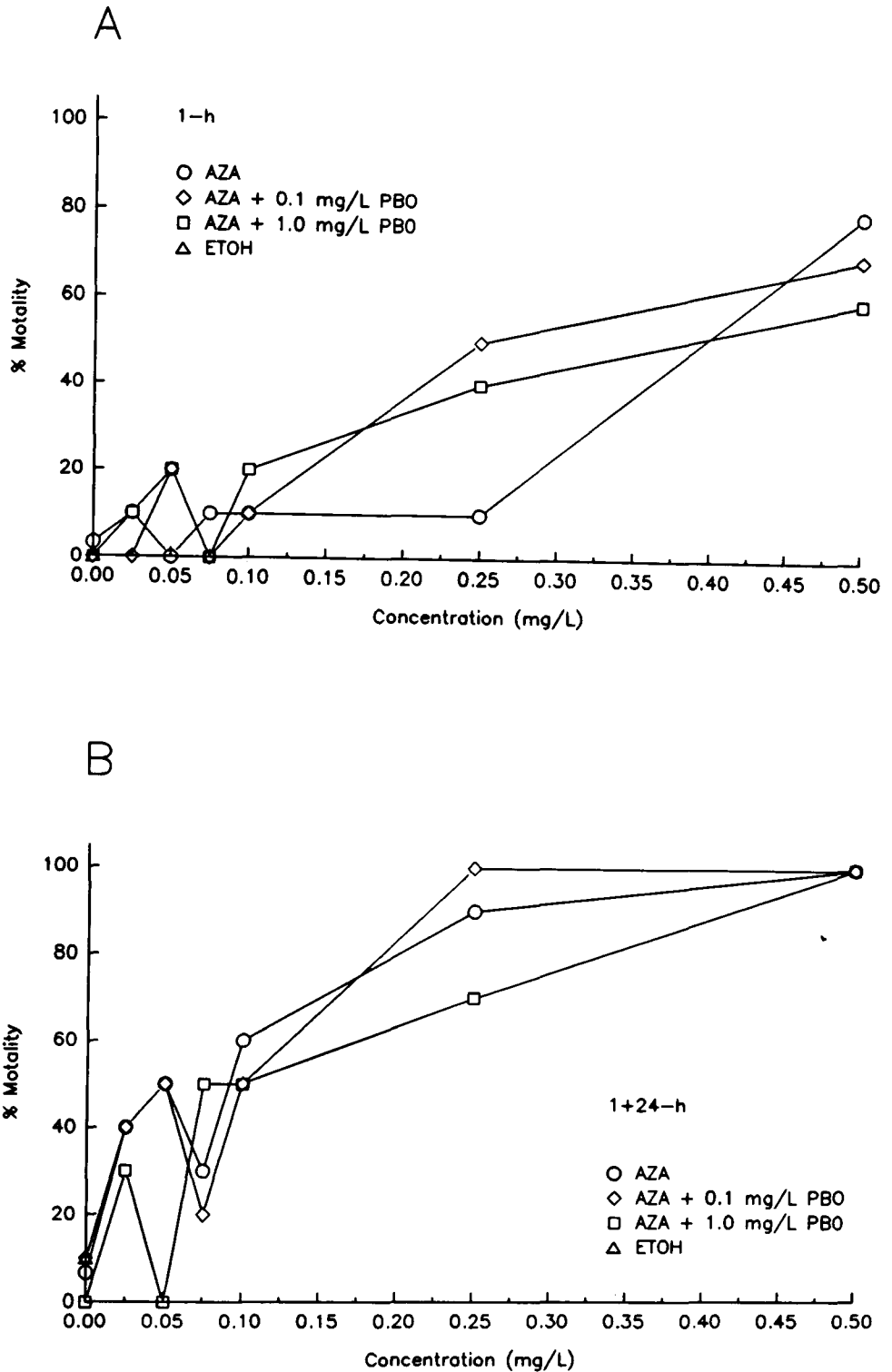concentrations of 0.1 mg/L resulting in a 40% mortality rate.
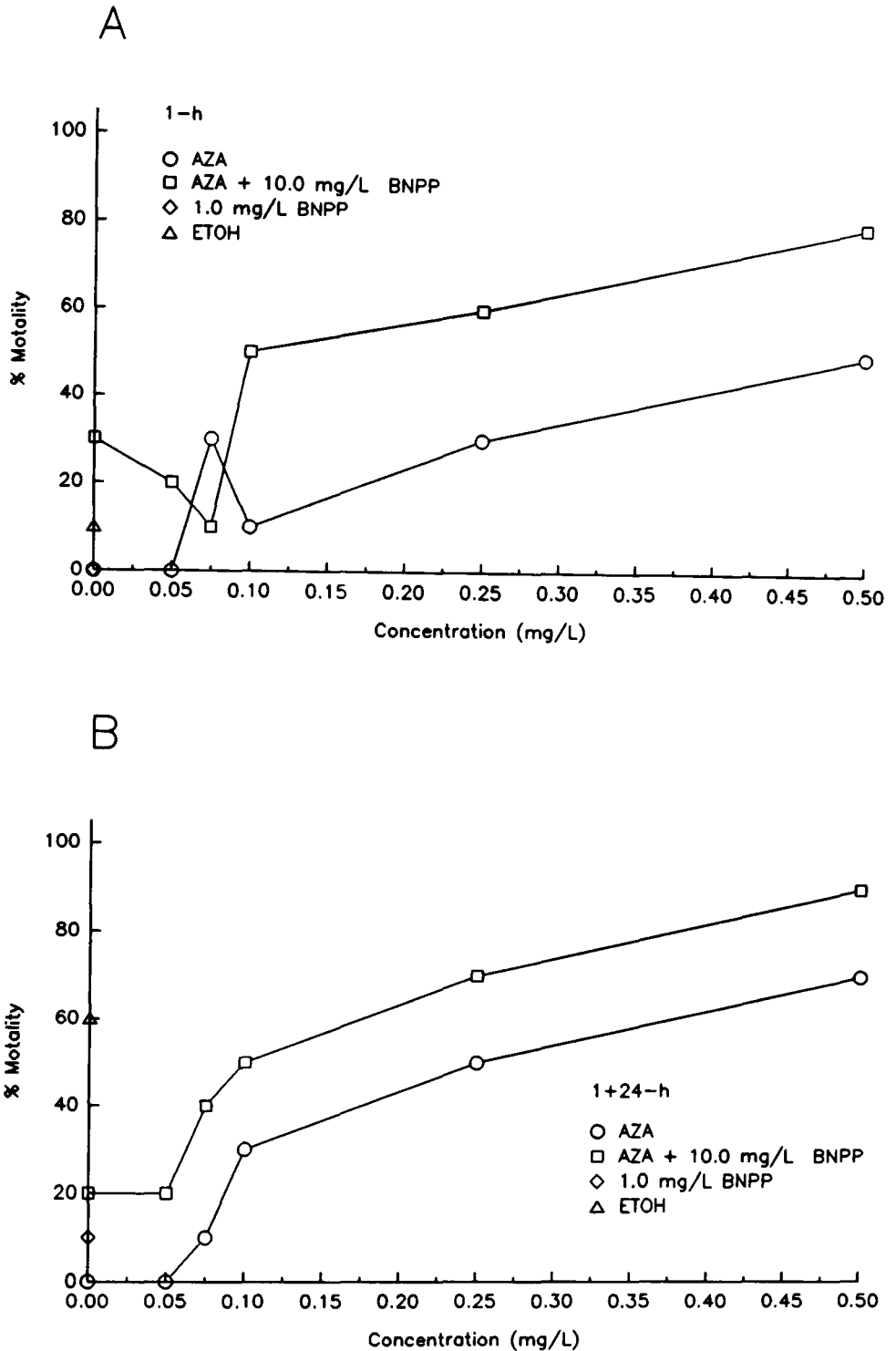
Figure 8.12. Acute toxicity of azamethiphos (AZA) and AZA + BNPP combinations to sea lice following 1 hour exposures *in vitro* (response from beginning of exposure - A 1-h; B 1+24-h).
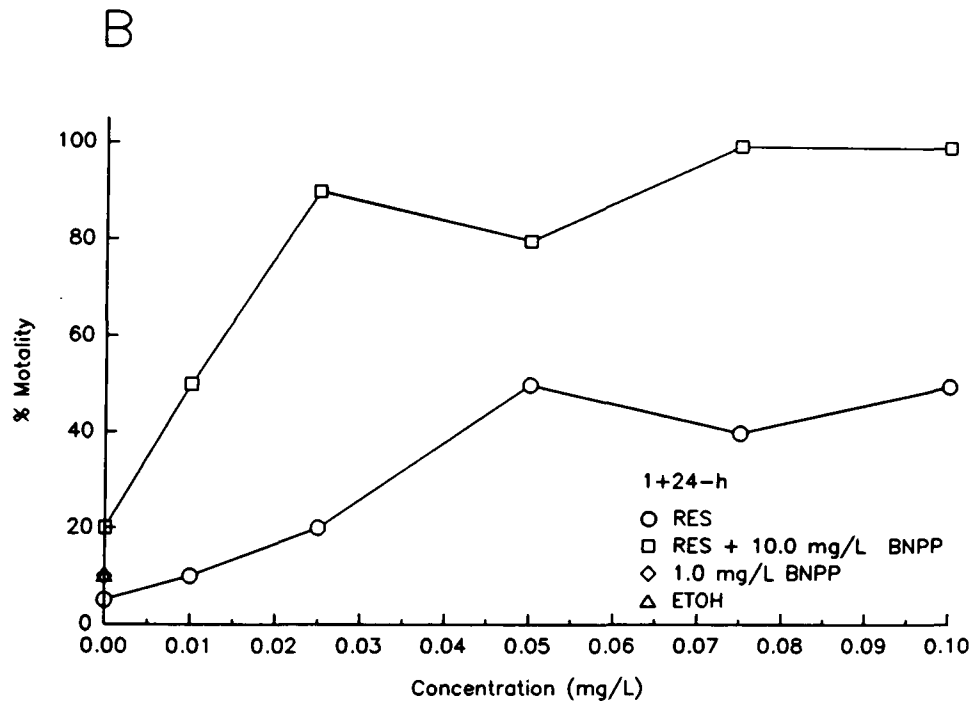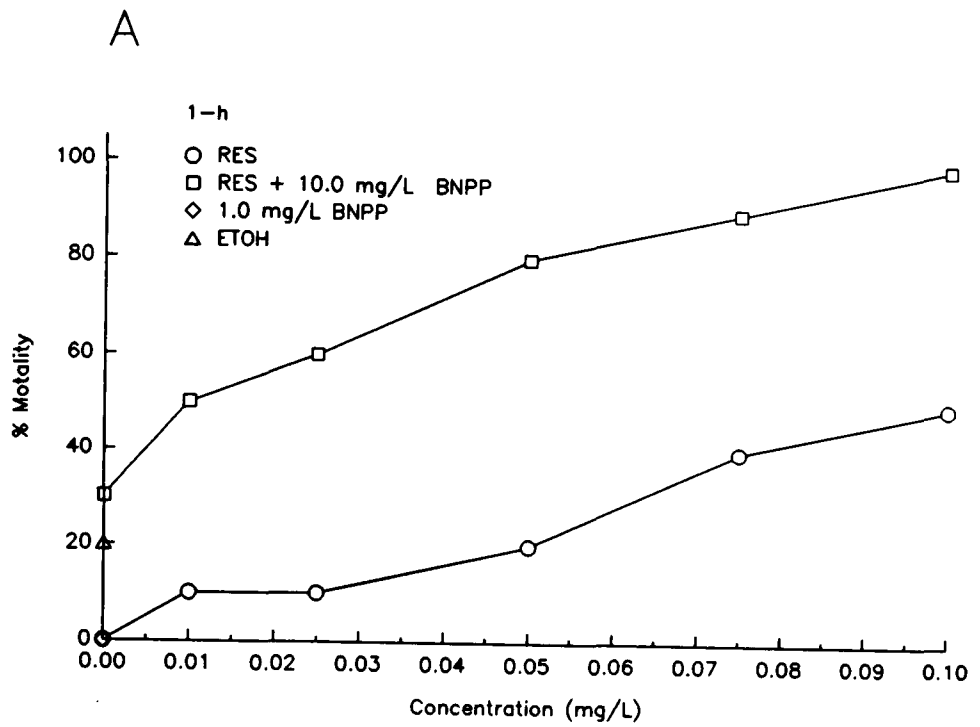
Figure 8.13. Acute toxicity of resmethrin (RES) and RES + BNPP combinations to sea lice following 1 hour exposures *in vitro* (response from beginning of exposure - A 1-h; B 1+24-h).

8.4 Discussion

The action of pesticides on target pests (e.g. insects) is dependant on either direct contact and absorption through the cuticle or by ingestion and subsequent absorption by the gut, also lined with cuticle. Thus, the effectiveness of a given pesticide is dependant on both the structure of the cuticle and the structure/chemistry of the pesticides. In caligid copepods, the cuticle is comprised of an epicuticle and endocuticle similar in structure to that of decapod crustaceans (Kannupandi 1976). However, the relative thickness and degree of hardening varies in different regions, with the thinnest, softest, cuticle being found in the intersegmental membranes. Furthermore, Kannupandi (1976) reported the presence of ducts extending from the dermal glands to the exterior.

In the present study [^{14}C]azamethiphos, which is moderately hydrophilic, first appeared in lice in the frontal plates and the first antenna, followed by diffuse accumulation in the cephalothorax and genital complex, suggesting uptake via passive diffusion. Accumulation in the frontal plates may suggest that cuticle associated with this structures are thin lined or porous (Kannupandi 1976), or may possibly serve a respiratory role. Alternatively, the compound may more readily penetrate sutures associated with the frontal plates (Kabata 1979) or via the filament gland organ which, in many species, is villiform and continuous with the underlying striated muscle (Oldewage & Van As 1989; Anstensrud 1990b; Pike, MacKenzie & Rowand in press).However, given the high degree of innervation associated with these structures (Wilson 1905; Laverack & Hull in press), it is possible that the presence of the labelled material may represent accumulation (rather than penetration) in areas rich in nervous tissue. Interestingly, there was no evidence of accumulation of label around the sub-

oesophageal ganglion (Walday & Fonnum 1989b). Walday & Fonnum showed that the sub-oesophageal ganglion was high in AChE activity, thus the lack exposure in this region may have been due to the relatively short range of ^{14}C low energy β emissions (Faires & Boswell 1981). Attempts to section labelled material (for higher resolution of compound accumulation in internal structures) prove unsuccessful (data not presented), thus it is possible that further areas of localization of pesticide are yet to be described. Interestingly, Walday & Fonnum (1989b), did not show a high degree of AChE activity associated with the frontal plates which may have been due to sensitivity of the staining system used. Thus it remains to be seen if the labelled material detected in the frontal plates was bound to nervous tissue (and therefore accumulated) or represented primary areas of absorption.

Given the slow rate of movement of labelled material into the hindgut from the anus, it is likely that uptake via the anus was passive and in contrast to the early observation by Gnanamuthu (1948), who reported that water did not actively move in and out of the anus, clearly shows that fluid can readily pass into the hindgut and may therefore suggesting a possible respiratory function. Other discrete openings in the integument include the pore canals and glands located in protopodites in the second and third legs (thoracic legs 3 & 4) (Scott & Scott 1913). Although some labelled material was observed to be associated with the second and third legs, the presence of label appeared to be associated with several areas of prominent armature (maxillipeds, 1st and 2nd thoracic legs), possibly due to areas of thin cuticulization associated with joints, or due to large surface areas associated with a high number of setae. In addition, a time dependant increase in the amount of labelled material appeared to occur along the lateral sutures of the cephalothorax and may also represent thin areas in the cuticle

(Kannupandi 1976).

The labelled material observed to be associated with the genital complex is believed to have entered via openings leading to the seminal receptacle (= vulva), or possibly the pores from which egg strings are extruded. The significance of accumulated label is unknown and, as described above, probably represents passive diffusion.

In both the time and concentration trials, the total amount of [^{14}C]azamethiphos absorbed was found to reach a steady state, or decrease at the maximum exposure times and concentrations respectively. Given the high toxicity of azamethiphos at 0.5 mg/L (present study), it is possible that a threshold narcotic effect concentration was surpassed and the lice were affected by toxic/narcotic effects (Chaudhry & Price 1990). In their studies on the uptake of [^{32}P]phosphine in the lesser grain borer, *Rhyzopertha dominica*, Chaudhry & Price (1990) showed that uptake was negligible in insects which had been 'knocked down' or killed, but that metabolism of the compound appeared to continue for a limited time. In the present study it might be speculated that as lice became narcotized from exposure to [^{14}C]azamethiphos, any passive or active uptake was interrupted, with active metabolism removing absorbed compound as demonstrated by lower amounts of label being detected at 0.5 mg/L than 0.4 mg/L. Thus, the threshold concentration in these studies is believed to be between 0.4 and 0.5 mg/L.

At a concentration of 0.5 mg/L [^{14}C]azamethiphos, uptake reached a steady state ($\approx$ 29 ng/louse) after 50 minutes exposure. Although the toxicity of the exposures was not examined, the results suggests that prolonged exposures do not appreciably increase the toxicity of azamethiphos to lice. These data confirm support the findings obtained

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earlier (Chapter V), which showed that extended treatments did not increase efficacy proportionally. Similar results were reported by Bond Robinson & Buckland (1969) on the uptake of phosphine gas in insects who demonstrated that uptake was dependent on external factors such as oxygen content rather than exposure concentration or duration.

In insects a primary mechanism for the resistance to pesticides is reduced uptake in resistant strains (Price 1981; 1984; Chaudhry & Price 1989; Chaudhry & Price 1990). Unfortunately estimation rates for [^{14}C]azamethiphos for both sensitive and resistant lice could not be estimated due to several practical constraints (stability and availability of the labelled compounds, and time), but should be considered in future pharmacological investigations to assess the role uptake plays in sea lice resistance.

The lice sensitivity trials showed that the lice population from Loch Spelve was less sensitive to both DDVP and azamethiphos, confirming the findings of Jones *et al.* (1992) on the development of resistance to DDVP, and the presence of side resistance to azamethiphos, reported in earlier chapters. However, it could not be demonstrated that lice from Loch Melfort showed higher sensitivity to resmethrin than lice from Loch Spelve, therefore not supporting the hypothesis that cross resistance between OPs and pyrethroids has developed concurrently. This is in contrast to earlier findings which suggested that lice from OP resistant populations might also show reduced sensitivity to pyrethroids (see Chapters 4 & 5). However, given the high toxicity of resmethrin to lice, the high degree of variation in the data may reflect difficulties in accurately assessing the toxicity of extremely low concentrations to lice *in vitro*.

Trials with the two metabolic inhibitors, PBO and BNPP were found to give

contrasting results. Interestingly, no effect was observed with PBO to all the compounds tested. Whether or not MFOs play a role in pesticide detoxification is dependant on the pesticide/organism in question. In combination with various pesticides, synergists which inhibit MFOs have been shown to increase the toxicity of pyrethroids (permethrin & cypermethrin) (Glickman *et al.* 1982; Singh & Agarwal 1986), rotenone (Fabacher & Chambers 1972) and carbaryl (Singh & Agarwal 1989). In addition mixed function oxygenases have also been shown to be actively involved in the metabolism of organochlorines (aldrin) (Burns 1976). Since no synergistic effects were observed with the pesticides tested in the present study, it may indicate that MFO activity is not a significant detoxification pathway for these pesticides or that higher concentrations of PBO were required (suggesting a lack of adequate absorption).

PBO inhibits mixed function oxygenases which function to detoxify a wide variety of foreign compounds (Neff 1985) and are found in teleost fish (Payne & Penrose 1975; Burns 1976; Payne 1977; Gerhart & Carlson 1978), elasmobranchs (Payne 1977), crustaceans (Payne 1977), oligochaetes (Payne 1977; Lee, Stolzenbach, Singer & Tenore 1981; Fries & Lee 1984) and echinoderms (Payne 1977). Although no synergistic effect was observed in lice, it cannot be concluded that MFOs are not present. As described above, the presence of MFO activity is widespread across many phyla, thus further work would be required to confirm the presence or absence of MFO activity in lice. Furthermore, although MFOs may not play a role in pesticide detoxification *per se*, they may, if present, act as markers to identify individual populations of lice under heavy stress from environmental pollutants or chemotherapeutants (Neff 1985; Payne 1976).

In contrast to PBO, BNPP was found to have a synergistic effect with both azamethiphos as well as resmethrin, suggesting that detoxification in lice may be due wholly, or in part, to non-specific esterases. In trout, ester hydrolysis has been shown to be involved in pyrethroid metabolism, but the slow rates observed are thought to account for higher relative toxicity to fishes (Glickman et al. 1981; 1982). Interestingly, Glickman et al. (1982) found that no potentiation occurred in trout treated with the esterase inhibitor tri-O-toly phosphate (TOTP), but as mentioned above, found that PBO did potentiate permethrin.

High levels of non-specific esterase detoxication enzymes are a well known resistance mechanism in insects (Bisset, Rodriques, Diaz, Ortiz, Marquetti & Hemmingway 1990; Peiris & Hemmingway 1990). Such a non-specific mechanism would support the hypothesis that cross resistance may exist since non-specific esterases have been shown to attack ester bonds in OPs, carbamates and pyrethroids (Sawicki 1979). However, in many instances, resistance is not confined to one mechanism, but is usually found in combination with other mechanisms (Sawicki 1975). Where reduced sensitivity to OPs occurs, reduced uptake, increase esterase activity and/or decreased target (AChE binding) sensitivity may singly, or in combination, confer resistance (Sawicki 1979). Thus, although BNPP may act to increase the toxicity of both azamethiphos and resmethrin, it may only suggest that both compounds are metabolized by a particular esterase or group of esterases but which are unrelated to those which act to confer resistance (Bisset et al. 1990). Alternatively, the mechanism involved may be independent of ester hydrolysis (i.e. insensitive acetylcholinesterase) (Bisset et al. 1990). Furthermore, the relative amounts of BNPP (10 mg/L) required were much higher than the relative amounts of pesticides, and in fact, resulted in low toxicity

rates. Thus it remains to be seen if lower concentrations such as 1.0 mg/L, or less, which did not appear to be toxic to lice, can potentiate azamethiphos or resmethrin. It is obvious that a great deal more work in this area is necessary before any firm conclusions can be made.

The first case of resistance by a parasitic crustacean was reported by Lahav *et al.* (1962). These authors showed that not only were some populations of the branchiurians *Argulus foliaceus* & *A. pelucidus*, resistant to the organochlorine (OC) lindane, but that a 85 fold decrease in sensitivity could be induced following 6 treatments. It was also shown that although the populations resistant to lindane were also resistant to the related OC endrin, resistant populations were highly sensitive to the OP malathion. However, although both lindane and endrin are members of the OC group of insecticides, lindane is somewhat different in structure and is therefore subclassified in the benzene hexachloride sub-group (BHC), whereas endrin is a cyclodiene derivative. This difference is significant since present data suggests that the mechanism of cyclodiene resistance is thought to be specific to the subgroup (Sawicki 1975). This would therefore suggest that the mechanism involved in *Argulus* resistance was a general non-specific mechanism, demonstrating the rapidity in which non-specific resistance mechanisms can develop between structurally different compounds.

Since OC pesticides often select similar resistance mechanisms as pyrethroids (namely "knock down resistance") (Sawicki 1975), it might be speculated that resistance to pyrethroids could developed with the same rapidity, in light of the finding that detoxification may involve a non-specific mechanism. Thus, given the need for chemotherapy in sea lice control and the potential for the use of pyrethroids, further

work in this area is warranted. It is also suggested that characterization of esterase activity might be considered as an alternative marker for the identification of discrete lice populations which may aid in the understanding of epidemiology of lice population with respect to site specific geography.

9   General Conclusions

Throughout the course of this study, several alternative chemotherapeutants were identified as potential replacements or additions to AQUAGARD SLT* for the control of sea lice in aquaculture, yet it is difficult to identify the most suitable due to several advantages and disadvantages associated with each of the compounds studied. At present, dichlorvos and trichlorfon (organophosphates) remain the principle compounds used for sea lice control. In addition to the organophosphates, ivermectin (avermectins) is used for sea lice control, but on a much more limited basis. Alternative compounds which are currently being evaluated as alternatives include pyrethrum (included with the pyrethroids) and hydrogen peroxide (a strong oxidising agent and therefore fundamentally different from the neurotoxins). Unfortunately, of the 4 types of chemotherapeutants, pesticides classes represented, only the organophosphate compounds have been licensed for sea lice control (Schlotfeldt, 1992), while the others have only been available on a restricted basis and until adequately licensed, and therefore made readily available, they cannot be considered as 'alternatives'.

The chemotherapeutic control of sea lice control can only be practical if it is developed as a strategic approach. Simply switching form one chemotherapeutic agent to the next will only increase the presence of resistance in sea lice populations and therefore reduce the already limited number of compounds available for control. This can only be achieved if several compounds, with different modes of action, are applied in a systematic and logical manner to reduce the selection pressure for a particular resistance mechanism (Conway & Comins 1979). Of the organophosphorus compounds studied, azamethiphos was found to be the most efficacious. Where lice are sensitive,

the compound is highly toxic to lice, and exhibits larger therapeutic ratios than for dichlorvos. Azamethiphos was found to be well tolerated by salmon with limited effects on AChE depression, unlike dichlorvos where AChE depression may be cumulative (Raverty, 1989). Since azamethiphos is highly unstable in solution (Worthing & Walker 1987), and has a week affinity for particulate organic matter (due to a low partition coefficient), it is not expected to accumulate in marine sediments or significantly impact the marine environment. Furthermore, half life estimates for azamethiphos in salmon flesh are equal to, or shorter than, dichlorvos (P. Dobson per. comm.), which would allow short withdrawal times following treatment. However, azamethiphos is not without several disadvantages, the most serious of which is the development of side resistance between dichlorvos and azamethiphos. Results from the field trials confirmed that lice sensitivity will be the most significant modulating factor in the efficacy of azamethiphos when used for sea lice control. Trials at Loch Seaforth clearly demonstrated that where lice populations are sensitive to azamethiphos it is highly efficacious. However, where lice show varying degrees of reduced sensitivity to organophosphates, as seen in the Loch Creran and Duich populations, efficacy of treatment will be dependant on both the specific level of sensitivity and accuracy in estimating treatment dose. Thus it is recommended that sea lice should be tested *in vitro* prior to treatment with azamethiphos (or other chemotherapeutants) to determine the sensitivity pattern and the treatment dose required. .

In addition to reduced sensitivity in selected populations of lice, another disadvantage to the use of azamethiphos is its lack of effect on larval stages of lice, which will necessitate short term repeat treatments. Although it is clear that chalimus are not as susceptible as adults to the toxic effects of azamethiphos, it is unknown whether

chalimus stages suffer from sublethal exposure which, if present, would contribute significantly to accelerating resistance (Coles & Roush 1992). This effect is further complicated by the resultant increased treatment frequency which is related to the rate at which resistance is selected (Barton 1983; Martin *et al.* 1984). Thus although azamethiphos has a high potential as a short term remedy, it is not completely efficacious in dealing controlling sea lice as a chronic disease.

The second main class of pesticides evaluated were the pyrethroids. Interestingly, both *in vitro* and *in vivo* trials indicated that emulsifiers appeared to have little effect on the toxicity of pyrethroids to sea lice suggesting that such additives may not be necessary in formulations used for sea lice control. This is very significant as the omission of such additional compounds from chemotherapeutant formulations will undoubtedly facilitate the licensing of drugs for sea lice control. In general, the pyrethroids were found to be excellent candidates as alternative sea lice chemotherapeutants. In many instances therapeutic ratios were found to be much larger for pyrethroids than dichlorvos and, in some instances azamethiphos, indicating the potential of this group of pesticides for sea lice control.

In general, pyrethroids are poorly water soluble, and have a high affinity for organic particulate matter (Worthing & Walker 1987; Tagatz, Stanley Plaia & Deans 1987). It is therefore difficult to predict how such compounds would behave in marine sediments (Roth *et al.* in press). Furthermore, the variable presence of organic loading in net pens may act to decrease the potency of the compounds in an unpredictable fashion (Chandler 1990). Despite these limitations, large therapeutic ratios would help to overcome the difficulties in estimated dose rate when using tarpaulins.

The finding that pyrethroids may be effective as an oral chemotherapeutant was unexpected and may prove valuable in the future development of sea lice chemotherapeutants. As discussed previously, an oral approach to sea lice control would be ideal, but compounds such as trichlorfon and dichlorvos are unsuitable due, primarily, to their high toxicity and instability in fish tissues. The high efficacy achieved with ivermectin in this regard has been due, primarily to persistence (see below) which makes it much less desirable when added to the sea directly or, as in the case of a feed additive, indirectly. Although the persistence of any given pyrethroid will depend on the molecule in question, it remains to be seen whether or not pyrethroids are more or less stable in the marine environment (and in particular marine sediments) than compounds such as ivermectin.

However, as with azamethiphos, the pyrethroid compounds also have several limitations with respect to sea lice control. As discussed above, a lack of effect on the chalimus stages, would mean that not only would bath treatments have to be repeated but that resistance may be selected very rapidly. Results on the presence of cross resistance were inconclusive, and due to the potential of pyrethroids as sea lice chemotherapeutants, the sensitivity of different populations of lice, with respect to OP sensitivity, needs to be addressed.

As mentioned previously, ivermectin has seen limited success as an oral chemotherapeutant for sea lice control. However, it should be noted that ivermectin is not licensed for use in salmon and is not expected to be despite its potential for sea lice control (Brewer 1991). Given the poor absorption characteristics of orally administered ivermectin in salmon (Høy *et al.* 1990) and the potential for the compound to persist in

marine sediments (Halley *et al.* 1989a), it is unknown how long the compound would persist below cages where is likely to be deposited, or what effect sediment bound residues would have on benthic marine fauna. The results from the present study and others (Palmer *et al.* 1987; O'Halloran *et al.* 1992; Smith *et al.* in press) have shown that ivermectin and the related milbemycins, SKB5 & SKB8, are acutely toxic to salmon. Thus, efficacy appears to be related to the persistence of low levels of compounds residing in fish tissues. As discussed earlier, such a treatment regimen (low dose rates over a protracted period), although initially efficacious, are highly conducive to resistance selection. The results presented herein have demonstrated that related compounds, such as the milbemycins tested, have similar toxic properties as ivermectin. Thus it remains to be seen if the toxicity of the avermectins is related to structure or stability and whether or not less stable forms of avermectins can be developed for sea lice control.

In most instances, primary screening was targeted towards identifying those compounds which were found to be more toxic than dichlorvos. However, it should be stressed that in most cases, toxicity is relative; it therefore follows that compounds which are highly toxic to lice would also be expected to be toxic to salmon. Thus, the information gained from such toxicity testing could be greatly improved by evaluating acute toxicity to fish to allow calculation of therapeutic ratios. Although limitations on resources prevented large scale toxicity testing to salmon (toxic dose response), therapeutic ratios could be estimated for several of the compounds following further testing *in vivo* for efficacy. However, this was only achieved where the toxic dose to salmon was close to the toxic dose to lice. Where therapeutic margins are increased, it is impractical to simultaneously evaluate therapeutic ratios. It is suggested that in the

future evaluation of chemotherapeutic agents for sea lice control, determination of the toxic dose response to both lice and salmon should be determined before evaluating efficacy.

The use of topical chemotherapeutic treatments for the control of fish ectoparasites has been a standard procedure for many years. The adaptation of dip or bath treatments in intensive aquaculture, although successful in many instances, has many drawbacks and few advantages. The principle advantage is that treatments, although labour intensive, can be carried out relatively quickly, and results obtained within 24 hours. Furthermore, where compounds such as dichlorvos, azamethiphos or H_2O_2 are used, short withdrawal periods would allow farmers to deal with infestations should they occur close to slaughter. However, given the variation in dosing which may occur fitting tarpaulins to cages and the resultant variation in efficacy, as demonstrated in the present study, compounds require relatively large therapeutic ratios (10 or more). Where skirts or open net pen treatments are used (i.e. without tarpaulins or skirts, Grave et al. 1991b), underdosing results. Such variable dose rates, which err towards underdosing, can only undermine the practical use of any chemotherapeutic agent when used topically and probably has contributed most to the development of resistance to dichlorvos (Conway & Comins 1979).

Where dosing is accurate, the therapeutic ratio of dichlorvos is approximately 5, thus there is a potential risk of overdosing fish, resulting in fish kills. It is possible that farmers might, in some instances, prolong treatments in order to ensure that they have been effective. However, the results from the present study indicate that pesticides appear to have a residual effect rather than knock down effect when used for sea lice

control, which is contrary to land based agricultural practices. This presents a problem for the farmer in that, following treatment, effectiveness cannot be estimated until the following day. If treatments are extended, there is a risk of toxicity. However, if treatments are not successful, then fish need to be treated a second time which would increase the toxicity risk, use more treatment compounds (thereby increasing cost & possible environmental effects) and promote resistance. In many of the azamethiphos field trials, it was thought that under dosing may have contributed to poor efficacy in several instances. One advantage to the use of H_2O_2 is that dose rates can be monitored on site with the aid of an on-site colourmetric test kit which allows accurate dosing (M. Jones pers. comm.).

Size of farm sites also presents problems with respect to topical treatments. Where cages are very large, such as with large offshore oceanic cages, the use of tarpaulins is impossible. However, even where smaller more manageable cages exist, the large numbers which may be present (60+) may require two or more weeks to effectively treat all the cages within a site. Given the susceptibility of fish to lice when they are stressed (Johnson & Albright in press), it is essential that as many fish are treated in as short a time span to reduce the overall parasite burden in a given site. Topical treatments undoubtedly stress fish, which would thereafter be predisposed to infection from the large reservoir of parasites infecting fish elsewhere on the farm.

The alternative to topical treatments, in a broad sense, is oral chemotherapy. However, due to the non-selective nature of neurotoxins required to kill ectoparasites, most compounds show toxic effects to the host before they have an effect on the external parasite. It is possible that compounds such as insect growth regulators or

feeding repellents which are selectively toxic to the parasites may prove useful. Oral chemotherapy is also complicated in fish due to poorly developed detoxification pathways. Furthermore, due to the large numbers of animals involved, it is often difficult to distribute the medicated feed evenly to ensure adequate dosing. Despite these limitations, effective oral chemotherapy has been demonstrated for sea lice control. Unfortunately, adding chemicals to the feed does not imply that compounds are not ending up in the sea in an active form. Compounds such as ivermectin and DFB which have been evaluated for sea lice control are poorly absorbed by fishes, thus data on the fate of such compounds will have to be addressed to allow adequate environmental assessment.

Although preliminary the results from the oral chemotherapy trials highlighted the potential of alternative treatment regimes, they also demonstrated the difficulties in using laboratory models for the evaluation of specific treatment protocols. Given the potential for oral therapy in sea lice control, further work will be necessary to asses the suitability of such laboratory models, as will research in a clinical setting, which at present is limited by availability of suitable (i.e. licensed) study sites.

The mechanisms responsible for resistance to organophosphates in lice are as yet unknown and, due to the implications resistance can have on reducing the number of available, efficacious, compounds for sea lice control, will require further research. It therefore follows that further work will also be required on the uptake and metabolism of organophosphates, and other potential alternatives, in lice to identify possible resistance mechanisms and possible measures to counteract them (Sawicki 1975). By identifying the mechanisms which confer resistance to a given chemotherapeutant,

adequate treatment strategies can be developed. Despite the urgent need for such fundamental information as uptake metabolism, this area of sea lice biology is the most neglected. Lastly, an understanding on the metabolism of chemotherapeutic agents will undoubtedly contribute to the development of more efficacious compounds which are selectively more toxic to lice.

In the past a lack of suitable management techniques has meant that chemotherapy was the principal method of sea lice control. With the development of wrasse technology and husbandry management techniques, such as "all in all out" policies and fallowing, chemotherapeutants will eventually become one component within a sea lice management strategy. However, the use of chemotherapeutants must also be developed in a strategic manner. Thus the availability of more than one chemotherapeutant and method of application is essential. For example, although ivermectin is very effective for lice control, long withdrawal times are observed on treated fish. Such long withdrawal times would conflict with harvest schedules, thus compounds such as azamethiphos would be desirable to deal with unexpected infections where fish were near to slaughter. Another treatment compound/application currently being developed which would complement existing strategies is the use of pyrethrum in conjunction with fish pumps (Vetrepharm 1992). One advantage of such an application is that fish can be treated when they are graded or otherwise handled. It would also allow the use of compounds at higher dose rates for shorter exposure periods benefitting from the residual action displayed by many pesticides. Such rapid immersions are not practical where tarpaulins are used due to the difficulty in handling them. Furthermore it would also be possible to develop technology which would not only treat fish but would contain the compound used for treatment (or most of it). However, since it is stressful

to grade fish, such treatments would not always be practical, as they predispose fish to furunculosis. Thus although such a treatment application may be beneficial in some instances, its potential would be maximized if it was used alongside alternative treatment methodologies which in turn would be based on different types of chemicals.

All of the potential anti-lice chemotherapeutic compounds discussed have advantages and disadvantages, as do the techniques used to deploy them. A strategic approach to lice control is necessary which employs more than one compound and treatment technique. When integrated into farm management techniques to counter lice infestations, such as fallowing, biological control and the possible use of vaccines, the use of chemotherapeutic compounds can be minimized thus maximizing their effectiveness for sea lice control. However, before such integrated management control strategies can be developed continued research will be necessary to assess the suitability of alternative chemotherapeutants.

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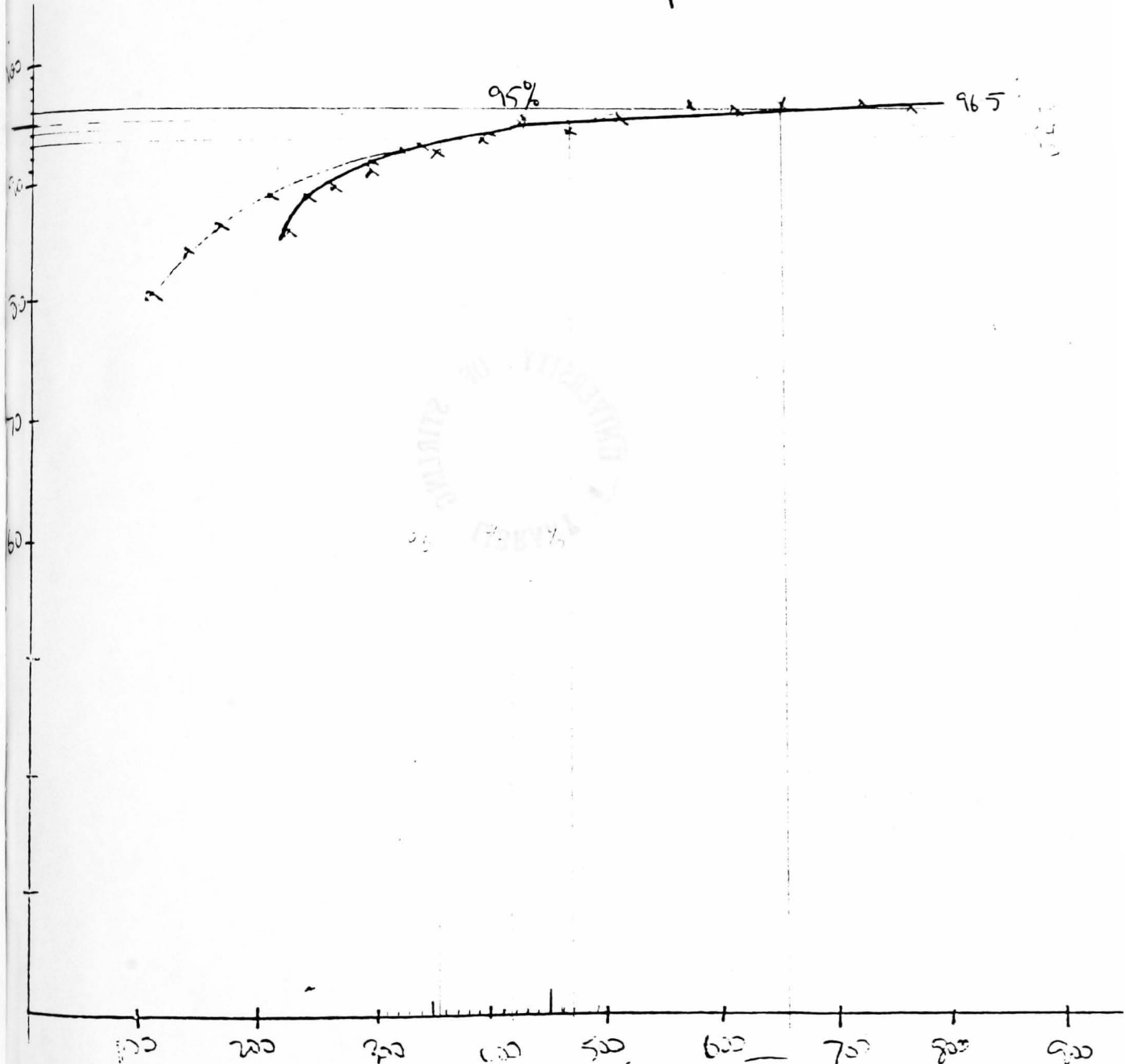
Appendix I

Quench correction plot for carbon-14

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Quench correction plot for carbon-14

Quench Correction.

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