Ursidae: The Undergraduate Research Journal at the University of Northern Colorado

Volume 5 | Number 3 Article 7

January 2016

# Joint Action Effects of Emamectin Benzoate and Cypermethrin on the Marine Copepod Tigriopus californicus

Lindsay Schultz

Michael Kelly

# Joint Action Effects of Emamectin Benzoate and Cypermethrin on the Marine Copepod *Tigriopus californicus*

**Lindsay Schultz and Michael Kelly**

**Faculty Mentor: Dr. Ginger Fisher, Biological Sciences**

This project examined the effect of two pesticides on the marine copepod *Tigriopus californicus.* The pesticides are emamectin benzoate and cypermethrin, which are commonly used in salmon aquaculture to treat the salmon for sea lice, a type of parasitic copepod. The results of this study indicate that these pesticides are toxic to the free-living *T. californicus* at very low dosage levels when used individually. When the pesticides are used in combination, the mortality rate is similar to when they are used individually, but there is an additional paralytic effect on the animals. All animals exposed to any of the pesticide mixtures used in this study were irreversibly paralyzed and therefore effectively killed. This indicates a strong need for more testing on the combined impacts of pesticides and increased regulatory action for salmon aquaculture.

**Keywords:** Pesticides, paralysis, pemamectin benzoate, cypermethrin, aquaculture, salmon, sea lice, Tigriopus californicus

Fishery stocks worldwide continue to decline due to increased fishery pressure and unsustainable management practices (Naylor *et al.,* 2000). One potential solution to the decline in fish species has been aquaculture, where fish and shellfish are farmed at high densities and harvested in large numbers to be brought directly to market. Landings from aquaculture have increased rapidly since the turn of the century and Bush et al. (2013) state that aquaculture now provides close to 50% of the world's seafood supply. One fishery in particular where aquaculture has increased dramatically is the salmon fishery, where over 1.8 million metric tons are produced annually (Burridge *et al.,* 2010).

Salmon are farmed in 24 countries, with Norway, Scotland, Chile and Canada leading the way as producers (Burridge *et al.,* 2010). In the United States there has been an increase in these farms along the Pacific Northwest and the coast of Maine (Shaw *et al.,* 2008). Salmon farms are typically located just offshore and consist of a number of large circular nets known as floating pens. Juvenile fish are placed into the floating pens to continue to grow to market size. Due to the high density of the fish in a relatively small space, parasites and diseases are a concern (Morales-Serna *et al.,* 2014). In response, salmon farmers have increased their use of pesticides to combat this issue. Pesticide use in the marine

environment has been increasing significantly in the past few decades due to a concomitant increase in these aquaculture activities, but the research to examine the effects and determine safe levels of these chemicals has been lagging (Morales-Serna *et al.*, 2014).

The purpose of this study is to examine two pesticides commonly used in salmon farming (cypermethrin and emamectin benzoate) to first determine their individual impact and then their impact when used together. The organism used to examine the toxicity of these pesticides is the marine zooplanktonic copepod *Tigriopus californicus*. Copepods such as *T. californicu*s are primary consumers and are considered the most common metazoans in the ocean (Feinberg and Dam, 1998). They are often used in toxicity studies due to their short life cycle and amenability to laboratory conditions. *Tigriopus californicus* is found in marine tidal pools throughout the Western coast of the United States, from Baja California to Alaska (Peterson, et al., 2013), and is common in areas where salmon farming is already occurring or proposed. We anticipate that this increase in salmon aquaculture will lead to an increase in the amount of pesticides discharged into marine tidal pools.

Both pesticides examined in this study, emamectin benzoate and cypermethrin, are typically used to treat sea lice, which are parasitic copepods in the family Calgidae (Morales-Serna et al., 2015). Emamectin benzoate is often administered orally, which allows the fish to have a direct source of the pesticide, without releasing it directly into the water. This form of administration causes no ill effects on the fish (Stone et al, 2002); however, it will be excreted in their feces (Sevatdal et al, 2005). This allows the pesticide to enter the marine environment, and can potentially move into tidal pools where species of non-parasitic copepods occur. Emamectin benzoate increases the permeability of muscle and nerve membranes to chlorine (Durkin, 2010), thus interfering with neurotransmission, causing irreversible paralysis (Anderson, et al. 2009). Emamectin benzoate has also been shown to change life cycle patterns, and egg production in aquatic invertebrates (Durkin, 2010). Although *T. californicus* is not the target organism for this pesticide, it is likely to be exposed to the chemical if it is used widely to treat sea lice in salmon farms along the western US coastline.

Cypermethrin, another pesticide commonly used in salmon farming, is a synthetic pyrethroid that has traditionally been used in terrestrial agriculture to control pests such as moths in cotton farms, fruit and vegetable crops. It has also been used as spot treatments for pests in stores, schools, and office buildings (Meister, 1992). However, cypermethrin has recently been used to control sea lice in salmon farming in a formulation called Excis (Gowland et al., 2002). The pesticide is added directly to the water in and around the sea cage where the salmon are held. Although cypermethrin is not readily absorbed into water, it has a high tendency to bind to particulate matter in the water (Gowland et al., 2002) and remain suspended for some time before settling into the sediments. (Muir, 1985; Agnihotri, 1986; Ayad, 2011). In invertebrates, cypermethrin is a neurostimulant that causes prolonged contraction of muscles and interrupts normal mobility (Willis, 2004). It has been found to damage the sodium channels, causing them to remain open for extended periods of time (Jones, 1990). This is why it has been used as an effective pesticide for sea lice, and why there is concern about its impact on non-target invertebrate species.

The Environmental Protection Agency, under the Federal Environmental Pesticide Control Act (FEPCA), requires

that toxicity testing be conducted before pesticides can be released into the environment (FEPCA, 1972). These tests are typically done by examining the effects of one pesticide at a time by measuring the dose of the pesticide that is lethal to 50% of the test animals ($LD_{50}$) in a defined time period, often 48 or 96 hours. While this type of assay provides useful information about the overall impact of the pesticide, it does not reflect the reality of pesticide exposure in the natural environment. Organisms are rarely exposed to only one chemical at a time, and in fact are often exposed to multiple chemicals simultaneously. The standard $LD_{50}$ tests do not reveal how these mixtures of chemicals will impact the species exposed to them. Because of this, it is crucial to determine how the interaction of chemicals can affect organisms. There are mathematical models that have been develop to attempt to predict the effects of the interactions of various chemicals (DeMarch 1987), but there have been few experiments conducted.

Because this study focuses on the mixture of the two pesticides, it was determined that we would be examining aspects of the joint action model (Bliss 1939). The joint action model examines how a mixture of chemicals can impact a particular species. Typically, if the mode of action of the chemicals is similar the concentration addition model applies (Anderson &Weber, 1975). In this case, the effect of the mixture would be the same as simply adding the same amount of each individually. However, if the mode of action of the chemicals is different, it is likely that the effect on the organism will match the response addition model (Anderson &Weber, 1975). Here, it assumed that each toxicant will have a threshold concentration before it causes a common response in the target species. However, in this model, it is more difficult to predict how the interaction of the chemicals will affect the target species, and what concentrations are needed to reach this threshold.

One mode of testing the joint action model is to use the toxic unit approach, where the toxic unit (TU), in this case the $LD_{50}$, is first determined and then the chemicals are mixed in ratios of their toxic units to assess their impacts on the target species. This can be best conceptualized by the equation $_{X}TU_{A} + {}_{Y}TU_{B} = 1\ TU_{(A\text{-}B)}$, where A and B are the two pesticides, x and y are the proportional toxic units of each pesticide, and TU is the $LD_{50}$ for each (Forget et al., 1999). If $x + y < 1$, the chemicals would be considered synergistic, thus, you have a lower TU when the chemicals are added in a mixture. If the two pesticides are shown to be synergistic in their effect, their overall impact is *more* than simply the addition of the two. If $x + y = 1$, then the chemicals are considered additive, which is indicative of the concentration addition model. In this case, it can be assumed that the toxicity of each pesticide compound can be summed to determine the maximum acceptable dosage; i.e. one can combine the $LD_{50}$ of each to arrive at an acceptable level. If $x + y > 1$, the chemicals are considered antagonistic, in that you have to add more to get the same response as 1TU, so the chemicals must be in some way counteracting one another. The goals of this study are to (1) to determine the toxicity level of cypermethrin and emamectin benzoate individually, and (2) to investigate whether combined exposure more closely matches the concentration addition model or response addition model and if the latter, are the pesticides synergistic or antagonistic.

## Methods

### Test Chemicals

A 95% pure formulation of cypermethrin (Sigma Aldrich) was dissolved in dimethyl sulfoxide (DMSO) to produce a stock solution that was diluted to final concentrations of 5 µg/L, 2.5 µg/L, .5 µg/L, and .25 µg/L. These concentrations were based on those of Willis and Ling (2004), who examined the effect of cypermethrin on other marine copepods. This process was repeated with a 95% pure formulation of emamectin benzoate (Sigma Aldrich), which was diluted to the same concentrations. Both chemicals will persist in solution for a time period longer than the 48 hour toxicity tests that were conducted in the study (Anderson et al., 2009; Muir et al,, 1995)

### Acute Toxicity Testing

*Tigriopus californicu*s (Carolina Biology Supply) cultures were maintained in 35ppt salinity at 20ºC (± 1.5) in a 12:12 light dark cycle and fed *Tetraselmis sp.* (Carolina Biological Supply) to excess. To determine the $LD_{50}$ values at 48h, 12 ovigerous females were placed individually into the wells of a 24-well plate and fed ad libitum for the duration of the exposure. For each toxicant concentration, at least three trials were conducted for a total of 36 ovigerous females per concentration. During the toxicity testing, animals were maintained at the same temperature, light cycle and feeding regimen as the stock cultures. Animals were examined at 48 hours and tested for their ability to move in response to gentle prodding with a probe. Any animal that exhibited a discernable response was considered to be alive at the end of the exposure. Due to the need to use DMSO to dissolve the pesticides, the impact of DMSO alone was also examined.

### Joint Action Effects

To determine if the effects of the pesticides were additive, the two pesticides were combined at 50% of their respective $LD_{50}$ values ($_{50}TU_{\text{emamectin benzoate}} + {}_{50}TU_{\text{cypermethrin}}$). Final concentrations were 1.28µg/L emamectin benzoate and 1.3µg/L cypermethrin. Thus, if the pesticides were additive, this should result in 50% mortality 48 hours after exposure. Again, 12 ovigerous females were individually placed into the wells of a 24-well plate. At the end of the 48 hour exposure, animals were examined under a stereomicroscope for their response to gentle prodding. These experiments were repeated twice.

To further elucidate the impact of the combined toxicants, and determine if there was a threshold response, copepods were exposed to varying levels of the pesticides. The first set of experiments exposed copepods to 75% of the $LD_{50}$ concentration for emamectin benzoate and 25% of the $LD_{50}$ concentration of cypermethrin ($_{75}TU_{\text{emamectin benzoate}} + {}_{25}TU_{\text{cypermethrin}}$), for final concentrations of 1.92µg/L of emamectin benzoate and 0.065µg/L of cypermethrin. Additionally, experiments were conducted 25% of the $LD_{50}$ concentration of emamectin benzoate and 75% of the $LD_{50}$ concentration of cypermethrin ($_{25}TU_{\text{emamectin benzoate}} + {}_{75}TU_{\text{cypermethrin}}$) for final concentrations of 0.64µg/L of emamectin benzoate and 1.95µg/L of cypermethrin. Three trials with 12 ovigerous females (for a total of 36 animals for each mixture) were conducted for each mixture and were examined for mortality after 48 hours using the methods described above.

### Recovery

To determine if the effects of the combined pesticides were reversible, recovery experiments were conducted. Following exposure to the 50:50 mixture, all copepods that were still alive were placed individually into wells containing only 35ppt seawater. After 2 hours and then again at 24 hours, animals were examined under a stereomicroscope to determine if they swam away in response to prodding with a probe.

### Probit Analysis

To calculate $LD_{50}$ values for each concentration, Probit Analysis was used. Probit analysis transforms a curved dose-response curve to a straight line that can be used to determine the concentration that is lethal to 50% of the population. Abbot's correction was used to correct for the mortality that was seen in the DMSO control (Rosenheim and Hoy, 1989)

## Results

### $LD_{50}$

Results from the acute toxicity tests of individual toxicants can be seen in Figure 1. From these data, Probit Analysis, with Abbott's correction for the effect of the DMSO, was used to determine the $LD_{50}$ values for each pesticide. The $LD_{50}$ for emamectin was 2.56 µg/L and the $LD_{50}$ value for cypermethrin was 2.6µg/L. These values appear slightly different than those in Figure 1 due to correction that was needed to account for the low levels of mortality that occurred in the DMSO controls.

### Joint Action Effects

When copepods were placed into the 50:50 mixture of emamectin benzoate and cypermethrin, all animals exhibited paralysis within 30 minutes of exposure. An animal was considered paralyzed when it was only capable of small twitching movements and exhibited no swimming or locomotory ability, even when prodded with a probe. At the end of the 48 hour experiment, an average of 47% of the animals were dead and all animals that remained alive were paralyzed (Table 1). Similar results were found with the other mixtures, in that all copepods were either paralyzed or dead at the end of the 48 hour exposure; however, the overall mortality decreased significantly in comparison to the mortality of the 50:50 exposure (Table 1 and Figure 2).

### Recovery

There was no evidence of recovery from paralysis for any animals exposed to the mixture of pesticides. This was true for both the 2 hour and 24 hour recovery times. It appears that the paralysis caused by the interaction of these two pesticides is not recoverable.

## Discussion

The data presented in this study indicated that both chemicals exhibit high toxicity at relatively low concentrations, even when copepods are exposed to a single pesticide. These data have important implications for the regulations of these chemicals. Presently, the recommended acute exposure limit for cypermethrin in drinking water is 940 µg/L (US EPA, 2006), and the $LD_{50}$ value for cypermethrin exposure to *T. californicus* found in this study is 2.6 µg/L, a far lower level. There are no comparable drinking water data available for emamectin benzoate, as this pesticide is still under review by the EPA and is not considered an impairment of bodies of water under the Clean Water Act (US EPA 2011). However, it is added

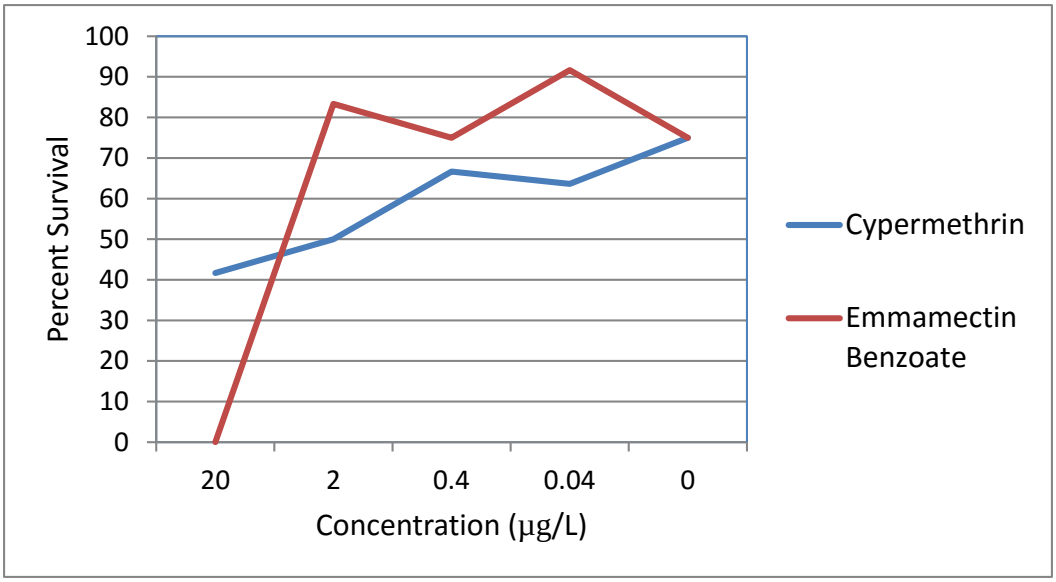


**Figure 1** Survival of *Tigriopus californicus* after 48 hours exposure to either cypermethrin or emamectin benzoate (n = 36 for each toxicant at each concentration).

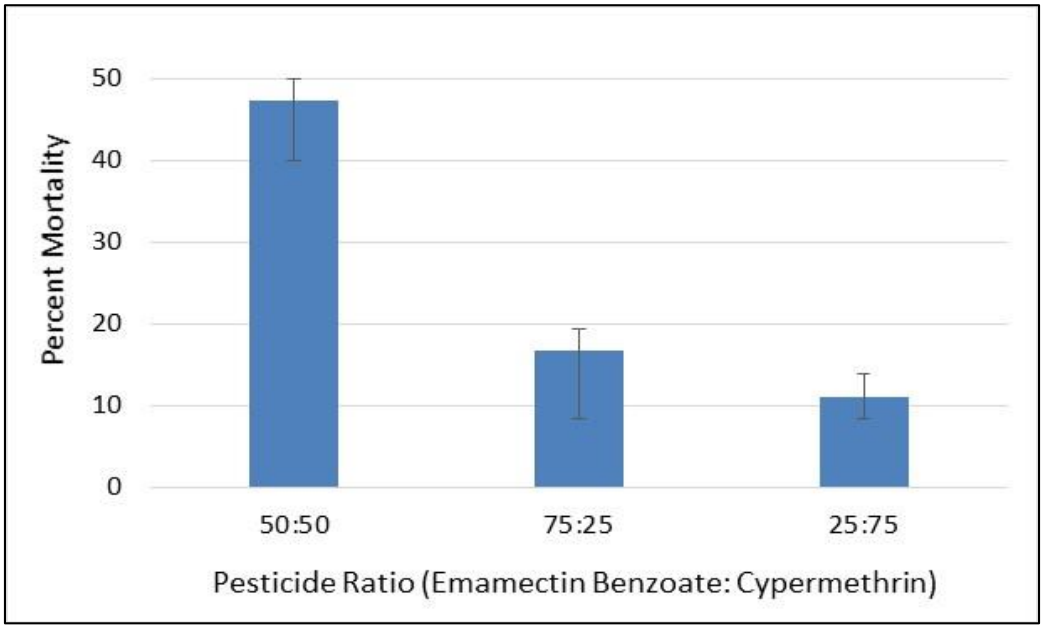


**Figure 2** The effect of pesticide mixtures on mortality of *T. californicus*. Ratios refer to the proportion of the $LD_{50}$ value for each pesticide. Asterisk indicates a significant difference ($p<0.05$) in mortality rate of the 50:50 ratio as compared to the others.

**Table 1** The effect of pesticide mixtures on *T. californicus*, including rates of mortality and paralysis. Ratios refer to the proportion of the $LD_{50}$ value for each pesticide.

| Pesticide Ratio (Emamectin Benzoate: Cypermethrin) | Average % Mortality | Average % Paralyzed | Total % Affected |
|---|---|---|---|
| **50:50** | 42.7 | 55.3 | 100 |
| **75:25** | 16.67 | 83.33 | 100 |
| **25:75** | 11.11 | 88.89 | 100 |

directly to water during treatments of salmon in salmon farms and levels of $4.16X10^{-6}$ µg/L have been recorded as long as 8 days after treatment (Willis and Ling, 2004).

While the data from the single exposure experiments indicate a need for more studies on the impact of these pesticides in the marine environment, the results from the mixture studies are even more concerning. When examining the joint action of these two toxicants, it appears that they do not fit well into either the concentration addition or the response addition model. Because both pesticides affect the nervous system of the target organism, and therefore have a similar mode of action, one could predict that they would follow the concentration addition model. If that were the case, the 50:50 mixture of pesticides should have resulted in a 50% mortality rate at the end of the 48h exposure. The data indicate that the mortality rate was 47.2%, which is very close to the expected value, but mortality rate alone does not paint the full picture of the effect of these toxicants. At the end of the 48h exposure, all copepods were dead or were completely paralyzed and this paralysis was not recoverable. Any copepod that is paralyzed and unable to swim or feed will not be capable of survival for more than a few days, and therefore the pesticide has effectively killed it, but will require a longer time to do so. Thus is appears that these pesticides act synergistically when presented in a mixture.

To further examine the response addition model and look for threshold concentrations, mixtures of 75% of the emamectin benzoate $LD_{50}$ to 25% of the cypermethrin $LD_{50}$ and 25% of the emamectin benzoate $LD_{50}$ to 75% of the cypermethrin $LD_{50}$ were tested. It appears that the mortality rate declines in these mixtures, but the overall paralysis effects remain the same, resulting in an eventual 100% mortality. So, while there appears to be a threshold effect of these toxicants in their ability to cause mortality, there is no threshold effect in their paralytic actions. We propose that this is evidence of the response addition model, in that these toxicants have slightly different effects on the neuromuscular system of the target species, and therefore the result is paralysis rather than mortality. The response addition model predicts that the interaction of two

toxicants can produce unexpected effects in the target species when combined, rather than simply an increase in mortality, which is what our data show.

If use of these pesticides becomes widespread, it could potentially devastate the native marine copepod community, which in turn will have a significant impact on the ecosystem as a whole due to the role that these organisms play in the marine food chain. A variety of tidepool fish, several genera of anemones, and some grapsid and pagurid crabs are known predators of *T. californicus* specifically (Dethier, 1980), and copepods in general are a major component of their ecosystem. They are significant grazers of the phytoplankton community, are important prey for pelagic fish species, and play a critical role in the microbial loop of the world's oceans (Turner 2004). Because of their role is prey items, bioaccumulation is a potential threat, but neither emamectin benzoate nor cypermethrin appear to bioaccumulate in the food chain (Chukwudebe et al., 1996). However, there is preliminary evidence that cypermethrin can accumulate in fish (Corcellas et al., 2015) and mussel (Gowland et al., 2002) tissue following exposure to cypermethrin in the water column. This has implications for the combined use of these pesticides in salmon farming and their impact on non-target species. The toxicity of both of these pesticides to copepods at the low levels found in the current study suggest that copepods may act as an effective indicator species for the health of the ecosystem as a whole. Based on the data presented here we argue that more testing needs to be done to determine the impact of these pesticides, especially their use in combination, on other aquatic species that are likely to be prevalent in areas where salmon farming occurs.

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