Ticks and tick-borne diseases surveillance:

Monitoring of acaricide resistance with the Larval Immersion Test in ticks collected from goats and cattle in the area of Mnisi (Mpumalanga), South Africa

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Date: September 2015

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Introduction

The main agricultural activity in the area of Mnisi, South Africa is livestock farming, of which goats and cattle account for a significant proportion of the total livestock population. The area has a sub-tropical climate, which in combination with high host densities, provides very suitable conditions for the survival and maintenance of ticks. Monitoring of tick infestations and the collection of data concerning tick-borne diseases are important prerequisites for the development of practical and meaningful tick control methods in the area.

The present study is a continuation of the work initiated by several students from Utrecht University, concerning the relative, seasonal and geographical abundance of tick infestations in relation to the prevalence of heartwater in goats in the Mnisi area. This study will now try to investigate the level of acaricide resistance these ticks might have developed in this area.

Ticks are a serious constraint to livestock farming in Mnisi (Mpumalanga), South Africa. Damage due to ticks can be reduced through the use of acaricides. In the area of Mnisi (Mpumalanga), South Africa acaricides are used on cattle, whereby macrocyclic lactones (amitraz), synthetic pyrethroids (cypermethrin) and organo-phosphates (chlorfenvinphos) are most frequently used.

Acaricide resistance is usually first recognized as a failure of treatment to eliminate tick burdens from cattle. Although failure of treatment is often the result of incorrect preparation or application of acaricides, the persistence of ticks after frequent, correctly applied treatments indicates that acaricide resistance is likely. [1]

There are three different types of resistance, known as acquired resistance, cross-resistance and multiple resistance. Acquired resistance is defined as resistance that results from heritable decreases in sensitivity to drugs with the passage of time. Cross-resistance is the sharing of resistance among different acaricides with a similar mode of action, and multiple resistance is a resistance to more than one drug, even though they have different modes of action. [2]

The rate at which acaricide resistance becomes established in the tick population is dependent upon many factors. [1] These factors can be divided into genetic, operational and biological factors. [2] Genetic factors include the frequency of the original mutation in the population before treatment and the mode of inheritance of the resistant allele (dominant, co-dominant or recessive). [1,2] Operational aspects of resistance development include the chemical nature of the drug, the possibility of cross resistance, drug persistence in the host and drug clearance kinetics. Also the frequency of acaricide treatment and the

concentration gradient of the acaricide are of interest. [1,2] Biological factors include generation time, offspring per generation and breeding patterns, host range and the proportion of the total tick population that is not exposed to the acaricide (also known as refugia). [1, 2]

Continued use of acaricides that kill ticks lacking resistance genes selects for individuals that do have them. Therefore, acaricide resistance is essentially time-compressed evolution. Acaricides do not cause resistance per se but they do contribute to the process by allowing the survival of resistant individuals. [3] This makes it clear that a thorough survey of tick susceptibility to the commonly used acaricides should be conducted to assess the presence of acaricide-resistant populations.

A lack of standardized techniques for diagnosing acaricide resistance appears to be the main difficulty in creating and maintaining tick resistance monitoring systems. [1] In selecting a suitable method for the assessment of acaricide resistance, the following test requirements must be met: The test should be sensitive enough to identify early stages of resistance development, it should cover the full range of chemical groups that are in use (including the newest active ingredients), the testing procedure should be simple and inexpensive and should also provide a rapid and reliable result and finally and most importantly it should be suitable for standardization among laboratories in various locations so the global monitoring and comparison of test results can be achieved. [1]

Unfortunately, none of the currently used tests meet all of the above requirements. Therefore improvement of protocols for diagnosis of acaricide resistance should be a continuing goal. In order to facilitate global monitoring and provide a basis for comparison of test results, standardized diagnostic methods should be adopted. In view of this and following the advice of experts since 1975, FAO has promoted the use of the standardized Larval Packet Test (LPT) for field investigations of acaricide resistance. [1] LPT is considered to be the most repeatable, although it is limited by the length of time that it takes. Hence it remains the test of choice for surveys and for definitive confirmation of a diagnosis of resistance. [1]

Although it is not recommended by the FAO, the Larval Immersion Test (LIT) has shown to be even more sensitive than the Larval Packet Test for diagnosing tick acaricide resistance. Preliminary results at CSIRO Australia, have shown that the LIT is much more sensitive than LPT and can be used for diagnosing resistance to a large variety of active ingredients. Comparative studies have also indicated SLIT results (Shaw Larval Immersion Test) can be compared with the Larval Packet Test (LPT) results as there is good agreement between results of the test methods. [2] For these reasons, the Faculty of Veterinary Science at the

University of Pretoria has chosen this method to assess the presence of acaricide-resistant populations in the area of Mnisi (Mpumalanga), South Africa.

The purpose of this study is to gain a better insight in the acaricide resistance level of ticks (*Rhipicephalus microplus*, formerly known as *Boophilus microplus* and *Amblyomma hebraeum)* in the Mnisi area by using the LIT.

Our aim is to collect base line data in order to create a better understanding of the susceptibility of the previously mentioned ticks to different classes of acaricides (macrocyclic lactones, organo-phosphates and synthetic pyrethroids) in the Mnisi area. The possible emergence of acaricide resistance has some important implications for the strategy and organization of tick control in the Mnisi area, and this paper will discuss some of the actions that should be undertaken.

Research questions

Main research question

What is the current level of acaricide resistance of commonly used acaricides in ticks (*Rhipicephalus microplus* and *Amblyomma hebraeum*) collected from cattle and goats in the area of Mnisi (Mpumalanga), South Africa?

Sub questions

- What can be done to delay the development of acaricide resistance in this area?
- What strategies of tick control regarding acaricide use would be recommended?

Materials and methods

In order to create valuable and comparable results, one of the most sensitive and most commonly used methodologies in South Africa, the Larval Immersion Test, will be applied. Since the ticks will be collected throughout the whole Mnisi area, no conclusions will be based on individual animals or households.

Study area

The study and tick collecting will be conducted in the Mnisi area, province of Mpumalanga, South Africa. This area covers about 29.500 hectare and is situated in the north-eastern corner of the Bushbuckridge Municipal Area. The area falls within the savannah ecosystem and in the Mpumalanga Province the life cycle of *A. hebraeum* continues throughout the year. Over 40.000 people are living in this region, surrounded by the adjacent Andover and Monyeleti provincial game reserves and the Kruger National Park. These residents are divided over an estimated 8555 households. [4]

The area is part of the Mnisi Community Programme, an initiative by the University of Pretoria and the Mnisi Traditional Authority. [5] Since this study is a continuation, the villages used for sampling will be the same as during the previous studies. The following villages located in the Mnisi area will be visited: Ludlow, Utha A, Utha B, Clare A, Clare B, Athol, Share, Gottenburg, Hlalakahle (Gottenburg B), Hluvukani, Welverdiend A, Welverdiend B, Thorndale, Dixie, Tlhavekisa, Shorty and Seville. (See Appendix A)

Study animals/population

A collection of fully engorged female ticks from goats and cattle at the 17 different villages will be carried out.

Tick collection

Ticks from sampled animals will be stored and transported in labelled containers until further analysis. The containers are preferably made of cardboard, with small holes which allow for enough air circulation and will be labelled with date and specific ID number. A special sheet will be composed with ID numbers for every sampled village. For every ID number the collection site (the name of the village, to keep track of geographical distribution), date of sampling, owners (if possible including house numbers), number of sampled goats and amount of ticks found on the individual goats will be recorded.

Acaricide resistance testing

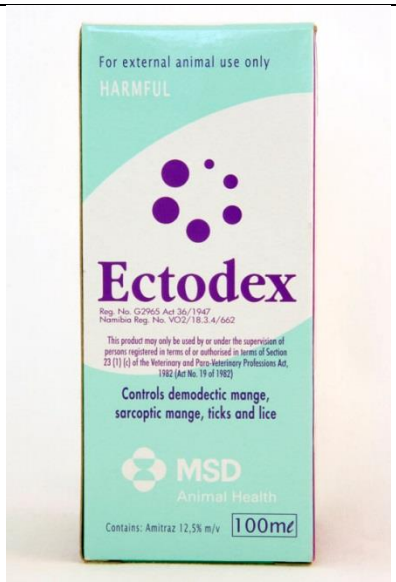
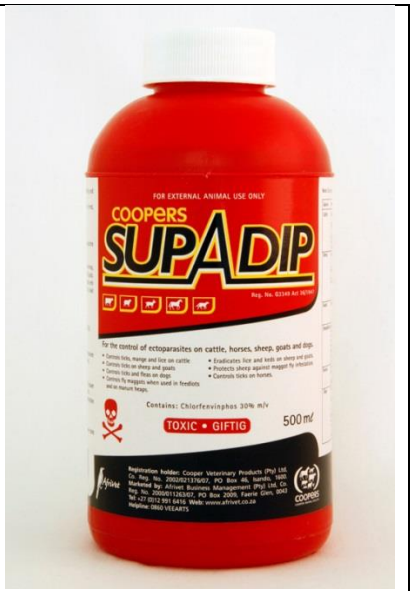
Depending upon their quality and availability, a carefully selected group of *R. microplus* and *A. hebraeum* ticks from as many villages as possible will be used for determining acaricide resistance. One of the most commonly used methodologies in South Africa, the LIT will be used.

Larval Immersion Test

After collection, the engorged female ticks will be kept at optimum circumstances (27 to 28°C and 80 to 95 percent RH) which will allow them to oviposit. The resulting larvae will be used for further testing. In this test, tick larvae are exposed to chemically impregnated filter papers (dipped) for exactly ten minutes and their subsequent mortality is quantified after 72 hours. [6] For a more detailed protocol description, see Appendix B.

Table 1 summarises the veterinary products that are most frequently used to control tick infestations in goats and cattle in the area of Mnisi (Mpumalanga), South Africa. These will therefore be used in this study.

Table 1: A list of the veterinary products that will be used for the LIT.

Product	 Ectodex®	 Ektoban®	 Supadip®
Marketer	MSD Animal Health	Novartis	Coopers
Active ingredient	Amitraz	Cypermethrin	Chlorfenvinphos
Chemical classification	Macrocyclic lactones	Synthetic pyrethroids	Organo-phosphates

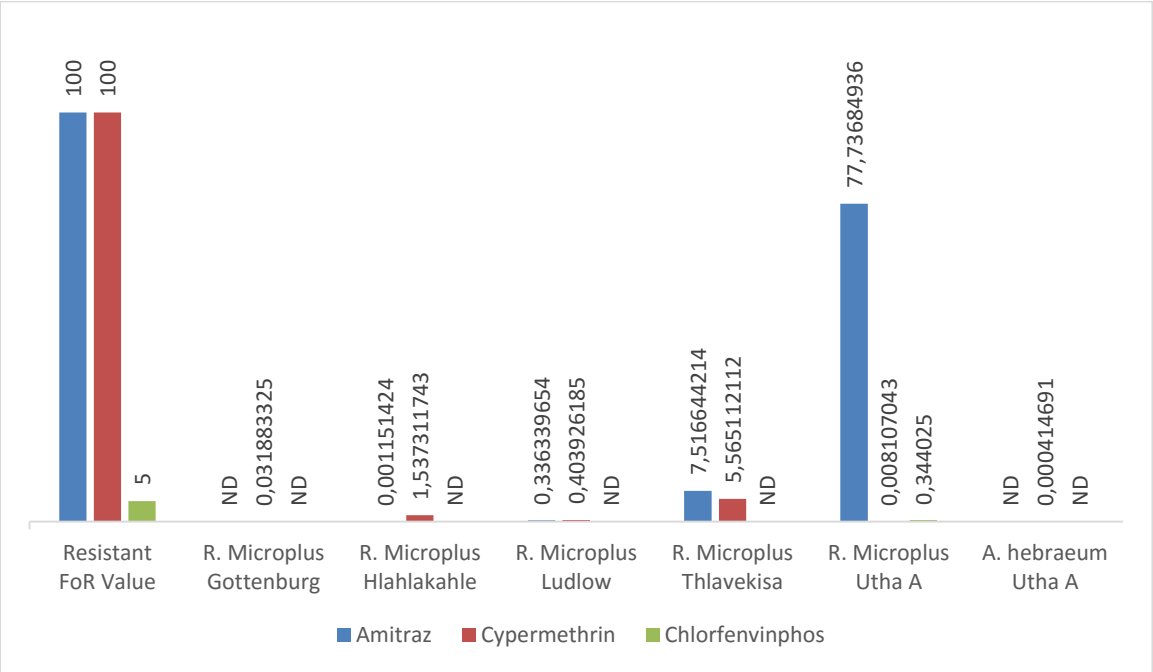
Results

Reference ticks:
The LC50, LC99 and Factor of Resistance (FoR) values for susceptible *R. microplus* and *A. hebraeum* strains (ticks collected from areas where the usage of acaricides were confirmed to be non-existent) had previously been determined by the Department of Veterinary Tropical Diseases of the Faculty of Veterinary Science at the University of Pretoria, and can be found in Appendix B.

Test ticks:
Due to the limited availability of larvae, only five populations of *R. microplus* (from the villages Gottenburg, Hlalakahle, Ludlow, Tlhavekisa and Utha A) and one population of *A. hebraeum* (from the village Utha A) could be tested. A summary of the LIT results, the corresponding log-probit concentration/CM % plots and subsequent LC50, LC99 and FoR determinations can be found in Appendix C.

Among all the tested tick populations no resistance to any of the tested acaricides could be detected. In a couple of tests the mortality was so high (100% mortality at every concentration) that the LC50, LC99 and FoR could not be determined and are therefore given the value 'ND' (Not Determined), as can be seen in Table 2 below.

Table 2: A representation of the Factor of Resistance values determined for the LIT tested tick populations in the area of Mnisi (Mpumalanga), South Africa.



The *R. microplus* population from Utha A seemed slightly less susceptible to amitraz and cypermethrin than the other tested populations (with lower mortality percentages at several concentrations, see Appendix C), indicating the possible emergence of developing acaricide resistance. Because of these results an additional 0-100% mortality range test for cypermethrin was executed. (See Appendix D) Unfortunately, we were not able to dilute the acaricide far enough to get the mortality all the way back to 0%. At the lowest concentration (0,00000000000000000262144) the CM% was still 36%. The dose mortality plot describes a heterogeneous susceptibility for cypermethrin in the *R. microplus* Utha A population.

Discussion

During this study, roughly 23.000 tick larvae from 5 different villages were examined for their susceptibility to acaricides with the Larval Immersion Test.

Among all the tested tick populations no resistance to any of the tested acaricides could be detected. The Factor of Resistance values all stayed below the developing/resistance values in all tests, indicating that the tested ticks are susceptible/ not resistant to acaricides with the active ingredients amitraz, cypermethrin and chlorfenvinphos.

The dose mortality plot of the 0-100% mortality range test describes a heterogeneous susceptibility of the tested ticks for cypermethrin in the *R. microplus* Utha A population. This indicates the presence of a mixed tick population with both higher and lower levels of cypermethrin resistance, which is commonly seen in field tick populations. The FoR values determined from these populations can therefore not fully indicate the true potential of the strain. [7]

Although there are currently no signs of acaricide resistance in the Mnisi area, preventive measures to delay resistance development in the future may prove to be worthwhile. A tick control strategy that minimises the selection of resistant genotypes could be of great value. [8]

To reduce the development of acaricide resistance, the knowledge gained by monitoring the different tick species and their resistance status to the different types of chemicals should be considered before the selection of acaricides. A possible strategy to delay the development of resistance would be to let farmers use acaricides with one specific active ingredient for as long as possible, all the while regularly monitoring for the possible emergence of acaricide resistance with laboratory tests like the LIT. Only once the test results indicate the development of resistance, one needs to change to an acaricide with another active ingredient. [1] Another approach for delaying acaricide resistance is the use of mixtures of different types of acaricides. This strategy is based on the likelihood that one individual tick will not have resistant alleles to multiple chemicals with different modes of action. This strategy was tested in South Africa and seems to have promising results. [1] Another variation on this approach is the acaricide rotation strategy, in which one alternates between two different kinds of acaricide. This stratey has also not yet been fully explored, but also seems to have some effects on the delay of acaricide resistance. [9]

One should keep in mind that the used acaricides should at all times be applied at the recommended concentrations, as little as possible and preferably only during tick season. Application of acaricides every 3 weeks during tick season is suggested in areas where tick

resistance is common. Because high frequency of acaricide application is a positive risk factor for the emergence of resistant strains, it is strongly advised that acaricide treatments should not exceed to more than five per season. [1,9]

None of these tick control strategies will do any good though unless they are executed properly. All of these tick control strategies will stand or fall by the quality of farmer education. Farmers in the Mnisi area should be educated regarding their knowledge of the proper use and handling of acaricides.

Finally, it is important to note that farmers should also be made aware of the fact that complete extermination of ticks can interfere with the endemic stability of tick-borne diseases. Overuse of acaricides could result in animals that are susceptible to heartwater and other endemic tick-borne diseases in this area. Therefore, any tick control programme should be aimed at the strategic extermination of ticks while attempting to maintain endemic stability to the diseases they transmit. [10]

Conclusion

As we failed to detect any resistance in the collected ticks to the most frequently used acaricides in the Mnisi area, we would like to conclude that the current level of acaricide resistance of commonly used acaricides in cattle and goat ticks (*Rhipicephalus microplus* and *Amblyomma hebraeum*) in the area of Mnisi (Mpumalanga), South Africa is low. The reports of tick control failures by farmers who controlled ticks with these products are therefore unlikely to indicate acaricide resistance.

In order to delay the development of acaricide resistance regarding acaricide use a sensible tick control strategy is required. This strategy would consist of various components, starting with regular monitoring of ticks for the possible emergence of acaricide resistance with laboratory tests like the LIT.

Different kinds of possible acaricide resistance delaying tick control strategies regarding acaricide use were mentioned. A possible approach would be to let farmers use acaricides with one active ingredient for as long as possible until it is no longer effective. Other approaches consist of using mixtures of different types of acaricide or the acaricide rotation strategy, in which one alternates between two different kinds of acaricide.

The final element of importance is farmer education. Farmers in the Mnisi area should be educated regarding their knowledge of the proper use and handling of acaricides, as well as their knowledge of the endemic stability of tick-borne diseases.

Acknowledgements

First of all I would like to gratefully acknowledge my research supervisors: faculty supervisor Prof. Dr. F. Jongejan, from the Utrecht Centre for Tick-borne Diseases (UCTD), FAO Reference Centre for Ticks and Tick-borne Diseases of Utrecht University, the Netherlands for allowing me to participate in this research project and external supervisor Dr. H. Stoltsz, from the Department of Veterinary Tropical Diseases of the Faculty of Veterinary Science, University of Pretoria, South Africa for supervising this research internship abroad. Their supervision and coordination of my work and advice on the writing of the research proposal and thesis are greatly appreciated.

I would like to thank Dr. Bjorn Reininghaus, the Limpopo Province state veterinarian, and Ms Jeanette Wentzel, the veterinary technologist and lab manager at the Hans Hoheisen Wildlife Research Station, for their great hospitality during my stay at HHWRS and much needed assistance during the preparations and carrying out of the Larval Immersion Tests.

Thanks are also due to Suzanne Busser, Annekim Geerdes and Laura Berger for their help with the planning, preparation and supervision of the laboratory work prior to traveling abroad.

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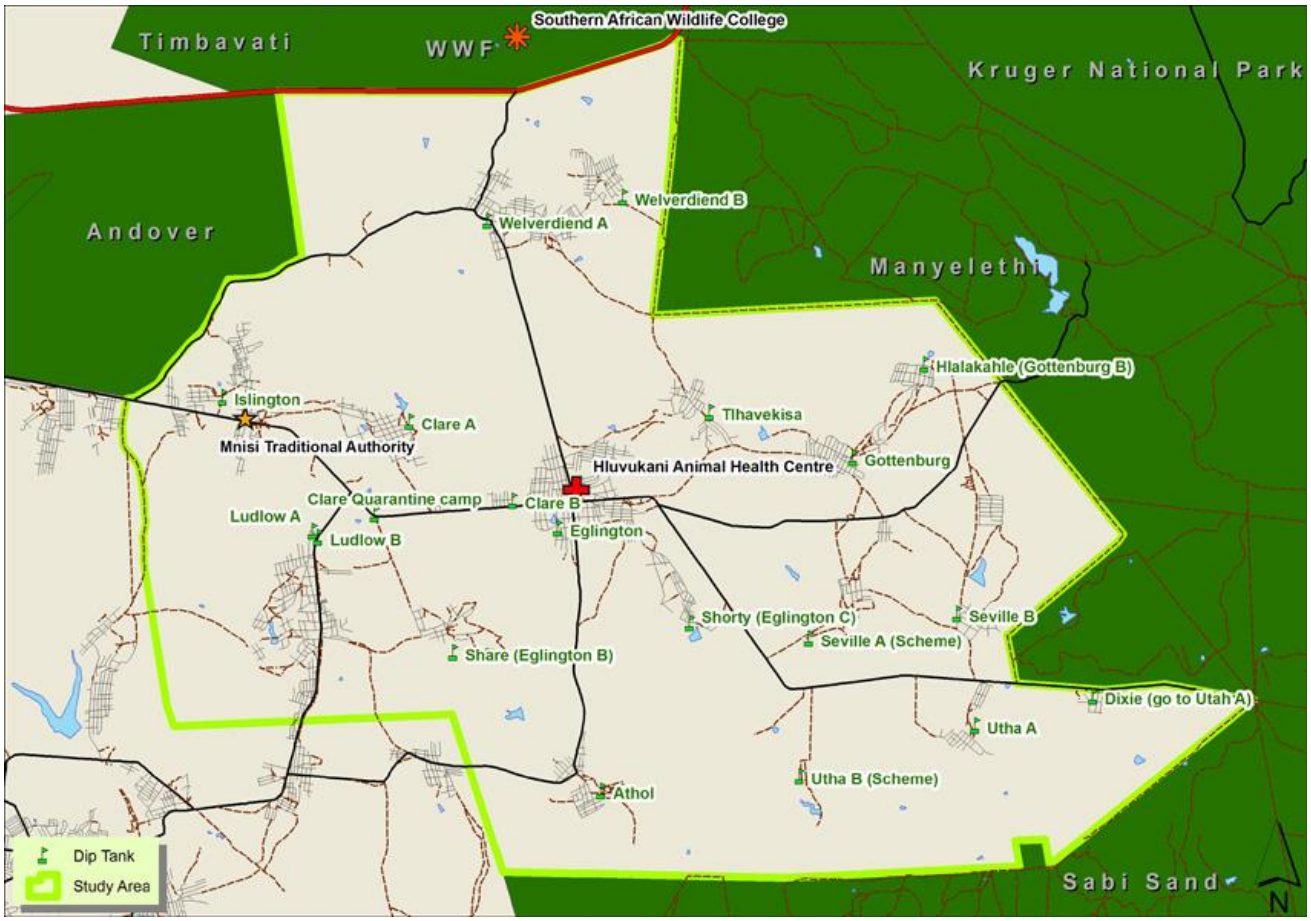
Appendices

Appendix A



Appendix B

PRACTICAL FOR THE SHAW LARVAL IMMERSION TEST [6]

Principle of the test

Shaw developed the larval immersion test in 1966 with the advantage that the larvae are immersed in a solution or suspension of the acaricide in order to increase toxicity. The Shaw LIT uses unfed larvae. Standardization of unfed larvae is more easily achieved than adult ticks and the mortality of the larvae can be recorded easily. Because the larvae are treated the same, the results are more credible statistically.

Collection

- Always collect as many undamaged, fully engorged ticks as possible, from as many different animals as possible.
- Engorged females greater than 4 mm only must be collected from animals before they are dipped, sprayed or treated with an acaricide.

Transport

- Ticks collected are to be placed in containers with perforated lids to allow circulation of air.
- Ticks should be placed between layers of paper towel in order to restrict movement and to absorb excess moisture.
- Do not transport ticks in airtight containers, plastic bags or glass tubes.
- Do not place the ticks in cotton wool.
- Do not expose the ticks or the transport medium to excessive heat or direct sunlight.
- Samples should be returned immediately to the laboratory for incubation.

Incubation of engorged female ticks

- Females from one sample location and one species are to be pooled and kept in an Erlenmeyer flask at 25 °C and relative humidity > 75% in an incubator for egg laying and hatching of larvae.
- Each flask should be clearly labelled with date of collection, species and sample location.
- Larvae are tested 18 to 21 days from hatching date. The date of hatching is determined to be when approximately 75% of larvae have hatched.
- Under optimal rearing conditions, the engorged female ticks of most species will begin to lay eggs within 2 to 7 days. The projected hatch date for Boophilus spp. (from the time engorged females are collected to the time of completion of 75% of larval hatch) is 40 days.

Laboratory handling

- The incubation conditions for all ticks before and during testing should be 27-28°C, 85-95% relative humidity.
- Packets are stored in the incubator at 25 °C and relative humidity > 75% and stacked in sequence on racks in such a way that they do not make contact. The water control packets are to be stored in a separate incubator.
- Mortality rates are determined 72 hours later, starting with the water control and lowest concentrations of acaricide.

Tick quality

- The engorged female ticks should be healthy (moving around and no damage and/or discoloration) and incubated as soon as possible after collection. Ticks below 4 mm in size should be rejected.
- The larvae used for the LIT should be healthy (moving around and no damage and/or discoloration).

Guidance notes before commencing a test

- At least two control packets and two for each of the concentrations per acaricide active ingredient (AI) are used for each tick sample suspected of having developed resistance.
- The control packets are always prepared first, followed by the acaricide packets, each of these series being handled in ascending order of concentration.
- The use of a white tray enables any accidentally fallen larvae to be seen and subsequently trapped on adhesive tape.
- The initial preparation for the test can be quite time consuming, so it is highly recommended that certain preparation be carried out timeously before the test is scheduled to start. Once you have started the actual test, you are under strict time constraints, so be sure to have everything you need ready.

<table>
<tr><th colspan="2">Test protocol - Preparation</th><th>Done</th></tr>
<tr><td>1</td><td>Folding filter paper packets:
Circle of filter paper is folded in half, then in half again to form creases.
The four creases can be held and pushed together to form packets.</td><td></td></tr>
<tr><td>2</td><td>Labelling filter paper packets:
Label the packets with sample location, species, acaricide, dilution and date of test.</td><td></td></tr>
<tr><td>3</td><td>Making up the master solution (1%):

$1\% = \text{required volume X required concentration} / \text{Concentration of acaricide}$

Note: When making up master solutions, make sure there is enough left over for the test. You may have to increase the master solution in order to leave about 30 ml for the test.</td><td></td></tr>
<tr><td>4</td><td>Making up the dilutions:
Using a felt-tipped pen, label plastic dishes with the acaricide and concentration they will contain and place them in a line in order of increasing concentration.
Starting with the master solution, measure out the appropriate amounts of water for each dish.
Measure the required amount of concentrate into the master solution and place on stirrer. Stir well.
Using the master solution, measure the required amount of concentration into each dish.

Serial dilutions:
<table>
<tr><th>Amitraz (12,5% m/v)</th><th>Cypermethrin (20% m/v)</th><th>Chlorfenvinphos (30% m/v)</th></tr>
<tr><td>0,000006</td><td>0,00002</td><td>0,00013</td></tr>
<tr><td>0,000032</td><td>0,00001</td><td>0,0004</td></tr>
<tr><td>0,00016</td><td>0,0005</td><td>0,0012</td></tr>
<tr><td>0,0008</td><td>0,002</td><td>0,003</td></tr>
<tr><td>0,004</td><td>0,01</td><td>0,01</td></tr>
<tr><td>0,02</td><td>0,05</td><td>0,03</td></tr>
<tr><td>0,1</td><td>0,2</td><td>0,1</td></tr>
<tr><td>1%</td><td>1%</td><td>1%</td></tr>
</table></td><td></td></tr>
</table>

Procedure done:

By: **On:** **Signature:**

Test protocol – Dipping the larvae		**Done**
1	Using a soft brush, transfer approximately 500 larvae to a filter paper placed upon a disposable aluminium foil plate.	
2	Draw up 10 ml of stirred clean water (control) or diluted acaricide (starting with the lowest concentration) up with a syringe and lay it on the side of the tray.	
3	5 ml are poured onto the ticks on the filter paper.	
4	Place a second filter paper on top of the first one containing the ticks, then pour the remaining 5 ml onto the filter paper sandwich.	
5	Set the filter paper sandwich aside for exactly 10 minutes.	
6	Repeat until all concentrations and duplicates are done.	

Procedure done:

By: **On:** **Signature:**

Test protocol – Packeting the larvae		**Done**
1	After the larvae have been dipped for exactly 10 minutes, the filter paper sandwich is opened and the papers are placed on dry paper towel to absorb excess moisture.	
2	Starting with the water controls/lowest concentrations, approximately one hundred larvae are then transferred from the papers into each of two dry, pre-folded conical filter paper envelopes (marked with sample location, species, acaricide, dilution and date of test) with a clean brush.	
3	Make sure the larvae are placed as close to the centre of the envelope as possible, then seal with tape. Repeat until all concentrations and duplicates are done.	
4	The sealed packets containing the larvae are then placed in a vertical position on a rack, which is placed in an incubator for 72 hours. Make sure the water control envelopes are stored in a separate incubator to prevent contamination.	

Procedure done:

By: **On:** **Signature:**

Results

After 72 hours of incubation, the packets are removed from the incubator. Starting with the water controls, the packets are opened and placed on a sheet of paper. Using a prodder, all the live tick larvae (i.e. the ones which run around) are then squashed, counting them while doing so. After this, the remaining dead larvae are counted and added up to get to the grand total of larvae.

Enter these figures into an Excel spreadsheet and calculate the percentage of mortality (%M) for each concentration.

$$M\% = Amount\ of\ dead\ tick\ larvae\ /\ Grand\ total\ of\ tick\ larvae$$

Tick larvae immersed in water only should have a mortality percentage below 10%. If the water control mortality is greater than 10% the test is discarded and repeated again. In cases where the percentage mortality is less than 10%, the % mortality for the concentrations is corrected by that figure and the graph plotted using these figures.

Corrected mortality, according to Abbott's formula:

$$CM\% = ((\%i - \%c)/\ (100 - \%c)) * (100/1)$$

Where CM% = corrected mortality
%i = % mortality in concentration i
%c = % mortality in water control

Plot concentration and CM% on log-probit paper to determine the LC50, LC99 and Factor of Resistance.

Interpretation

The Factor of Resistance is calculated as follows:

$$FoR = (LC50/\ (LC99\ of\ acaricide\ read\ from\ graph))/((LC50)/(LC99\ for\ susceptible\ Strain))$$

LC50 and LC99 for susceptible *R. microplus* strains:

Acaricide	LC50	LC99
Amitraz	0,00001	0,0002
Cypermethrin	0,00002	0,0002
Chlorfenvinphos	0,00063	0,0018

LC50 and LC99 for susceptible *A. hebraeum* strains:

Acaricide	LC50	LC99
Amitraz	0,0001	0,0016
Cypermethrin	0,000015	0,00013
Chlorfenvinphos	0,00052	0,001

Susceptible and resistant Factor of Resistance values for *R. microplus* and *A. hebraeum* species:

Acaricide	Susceptible	Developing	Resistant
Amitraz	< 100		> 100
Cypermethrin	< 100		> 100
Chlorfenvinphos	< 3	3 – 5	> 5

Larval Immersion Test Results

Tick species on which tests are conducted:	
Location on which samples were taken:	
Test date:	Read date:

Product name: **Water control**								Active ingredient: **Water**	
Water control	**Test a**			**Duplicate test b**			**Calculations**		
	Total	**Alive**	**Dead**	**Total**	**Alive**	**Dead**	**M %**	

Product name:								Active ingredient: **Amitraz (12,5% m/v)**	
Concentrations	**Test a**			**Duplicate test b**			**Calculations**		
	Total	**Alive**	**Dead**	**Total**	**Alive**	**Dead**	**M %**	**CM %**	
0,000006									
0,000032									
0,00016									
0,0008									
0,004									
0,02									
0,1									

Product name:								Active ingredient: **Cypermethrin (20% m/v)**	
Concentrations	**Test a**			**Duplicate test b**			**Calculations**		
	Total	**Alive**	**Dead**	**Total**	**Alive**	**Dead**	**M %**	**CM %**	
0,00002									
0,00001									
0,0005									
0,002									
0,01									
0,05									
0,2									

Product name:								Active ingredient: **Chlorfenvinphos (30% m/v)**	
Concentrations	**Test a**			**Duplicate test b**			**Calculations**		
	Total	**Alive**	**Dead**	**Total**	**Alive**	**Dead**	**M %**	**CM %**	
0,00013									
0,0004									
0,0012									
0,003									
0,01									
0,03									
0,1									

Appendix C

Larval Immersion Test Results of *R. microplus* Gottenburg population

Tick species on which tests are conducted:	*Rhipicephalus microplus*
Location on which samples were taken:	**Gottenburg**
Test date: 02-12-2014	Read date: 05-12-2014

Product name: **Water control**					Active ingredient: **Water**				
Water control	**Test a**			**Duplicate test b**			**Calculations**	
	Total	**Alive**	**Dead**	**Total**	**Alive**	**Dead**	**M %**	
	212	198	14	234	224	10	5	

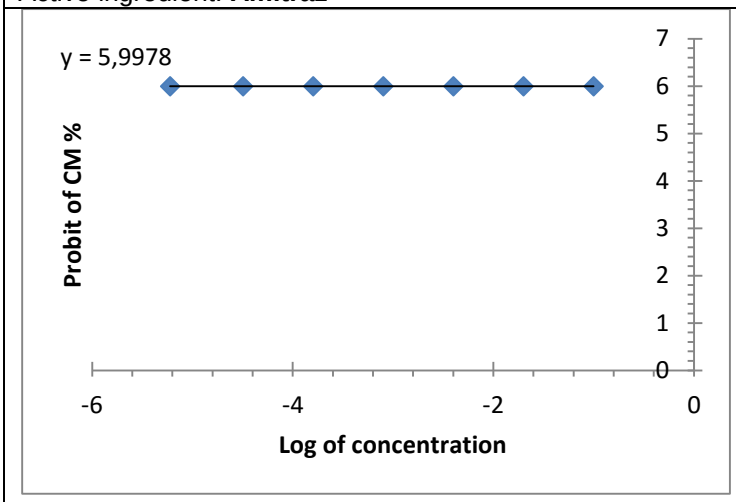
Product name: **Ectodex**					Active ingredient: **Amitraz (12,5% m/v)**			
Concentrations	**Test a**			**Duplicate test b**			**Calculations**	
	Total	**Alive**	**Dead**	**Total**	**Alive**	**Dead**	**M %**	**CM %**
0,000006	72	0	72	68	0	68	100	100
0,000032	80	0	80	73	0	73	100	100
0,00016	26	0	26	41	0	41	100	100
0,0008	74	0	74	25	0	25	100	100
0,004	46	0	46	37	0	37	100	100
0,02	38	0	38	48	0	48	100	100
0,1	53	0	53	66	0	66	100	100

Product name: **Ektoban**					Active ingredient: **Cypermethrin (20% m/v)**			
Concentrations	**Test a**			**Duplicate test b**			**Calculations**	
	Total	**Alive**	**Dead**	**Total**	**Alive**	**Dead**	**M %**	**CM %**
0,00002	235	0	235	192	1	191	99,77	99,75
0,00001	97	0	97	85	0	85	100	100
0,0005	48	0	48	69	0	69	100	100
0,002	17	0	17	23	0	23	100	100
0,01	20	0	20	16	0	16	100	100
0,05	43	0	43	43	0	43	100	100
0,2	54	0	54	48	0	48	100	100

Product name: **Supadip**					Active ingredient: **Chlorfenvinphos (30% m/v)**			
Concentrations	**Test a**			**Duplicate test b**			**Calculations**	
	Total	**Alive**	**Dead**	**Total**	**Alive**	**Dead**	**M %**	**CM %**
0,00013	56	0	56	82	0	82	100	100
0,0004	74	0	74	63	0	63	100	100
0,0012	73	0	73	64	0	64	100	100
0,003	48	0	48	76	0	76	100	100
0,01	38	0	38	79	0	79	100	100
0,03	31	0	31	25	0	25	100	100
0,1	38	0	38	61	0	61	100	100

Probit CM % log concentration plots of *R. microplus* Gottenburg population

Active ingredient: **Amitraz**

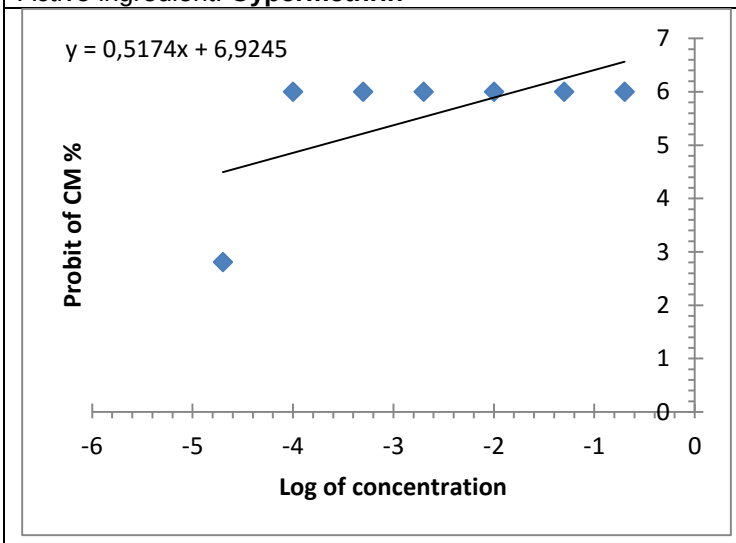


Equation y = ax + b	
a	**b**
0	5,99780702

LC	
LC50	**LC99**
Could not be determined	

Factor of Resistance
Could not be determined

Active ingredient: **Cypermethrin**

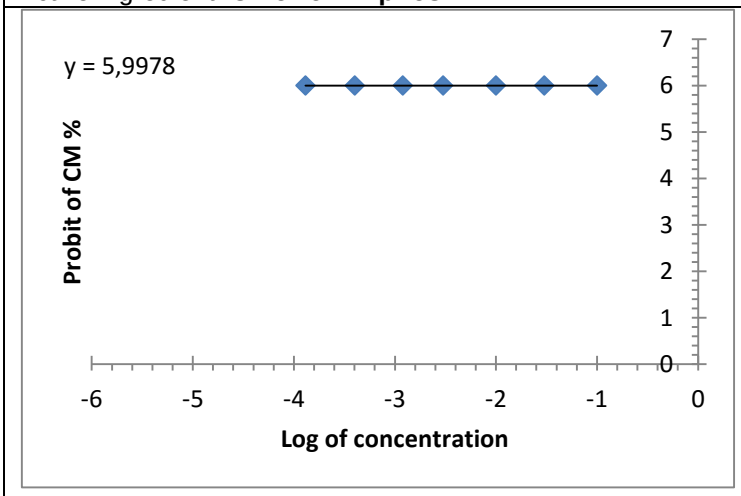


Equation y = ax + b	
a	**b**
0,517375911	6,924499163

LC	
LC50	**LC99**
$4{,}13158 \times 10^{-14}$	$1{,}29584 \times 10^{-9}$

Factor of Resistance
0,031883325

Active ingredient: **Chlorfenvinphos**



Equation y = ax + b	
a	**b**
0	5,99780702

LC	
LC50	**LC99**
Could not be determined	

Factor of Resistance
Could not be determined

Larval Immersion Test Results of *R. microplus* Hlalakahle population

Tick species on which tests are conducted:	*Rhipicephalus microplus*
Location on which samples were taken:	**Hlalakahle (Gottenburg B)**
Test date: 02-12-2014	Read date: 05-12-2014

Product name: **Water control**					Active ingredient: **Water**				
Water control	**Test a**			**Duplicate test b**			**Calculations**	
	Total	**Alive**	**Dead**	**Total**	**Alive**	**Dead**	**M %**	
	212	198	14	234	224	10	5	

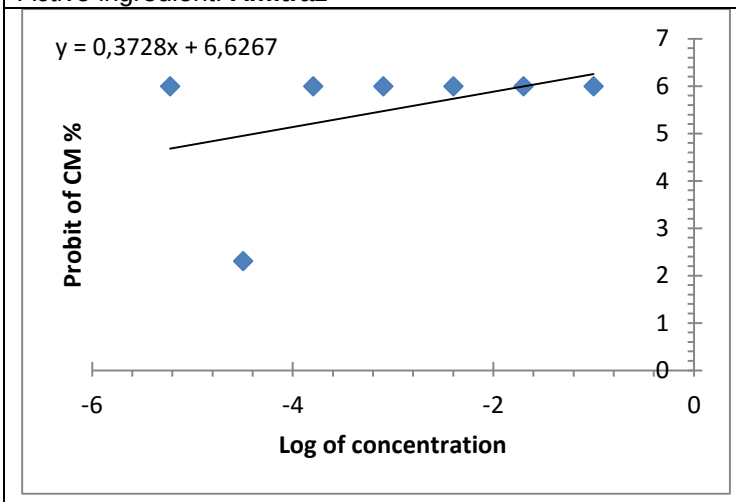
Product name: **Ectodex**					Active ingredient: **Amitraz (12,5% m/v)**			
Concentrations	**Test a**			**Duplicate test b**			**Calculations**	
	Total	**Alive**	**Dead**	**Total**	**Alive**	**Dead**	**M %**	**CM %**
0,000006	232	0	232	43	0	43	100	100
0,000032	70	0	70	131	2	129	99,00	98,95
0,00016	23	0	23	22	0	22	100	100
0,0008	106	0	106	85	0	85	100	100
0,004	84	0	84	59	0	59	100	100
0,02	116	0	116	98	0	98	100	100
0,1	53	0	53	51	0	51	100	100

Product name: **Ektoban**					Active ingredient: **Cypermethrin (20% m/v)**			
Concentrations	**Test a**			**Duplicate test b**			**Calculations**	
	Total	**Alive**	**Dead**	**Total**	**Alive**	**Dead**	**M %**	**CM %**
0,00002	91	2	89	82	2	80	97,69	97,56
0,00001	75	0	75	44	0	44	100	100
0,0005	94	0	94	110	1	109	99,51	99,48
0,002	52	0	52	56	0	56	100	100
0,01	37	0	37	48	0	48	100	100
0,05	43	0	43	88	0	88	100	100
0,2	163	0	163	71	0	71	100	100

Product name: **Supadip**					Active ingredient: **Chlorfenvinphos (30% m/v)**			
Concentrations	**Test a**			**Duplicate test b**			**Calculations**	
	Total	**Alive**	**Dead**	**Total**	**Alive**	**Dead**	**M %**	**CM %**
0,00013	48	0	48	125	0	125	100	100
0,0004	86	0	86	47	0	47	100	100
0,0012	48	0	48	53	0	53	100	100
0,003	27	0	27	34	0	34	100	100
0,01	24	0	24	30	0	30	100	100
0,03	28	0	28	73	0	73	100	100
0,1	25	0	25	54	0	54	100	100

Probit CM % log concentration plots of *R. microplus* Hlalakahle population

Active ingredient: **Amitraz**

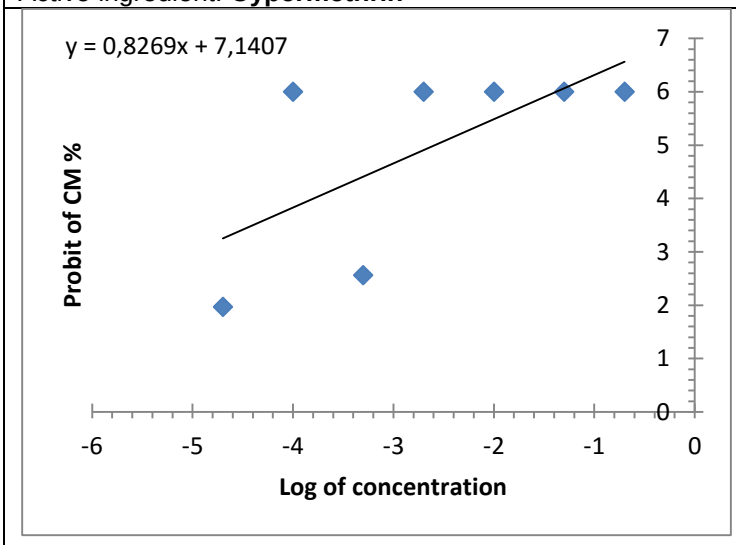


Equation y = ax + b	
a	**b**
0,372824414	6,626703152

LC	
LC50	**LC99**
1,68141 x10^{-18}	2,92057 x10^{-12}

Factor of Resistance
0,001151424

Active ingredient: **Cypermethrin**

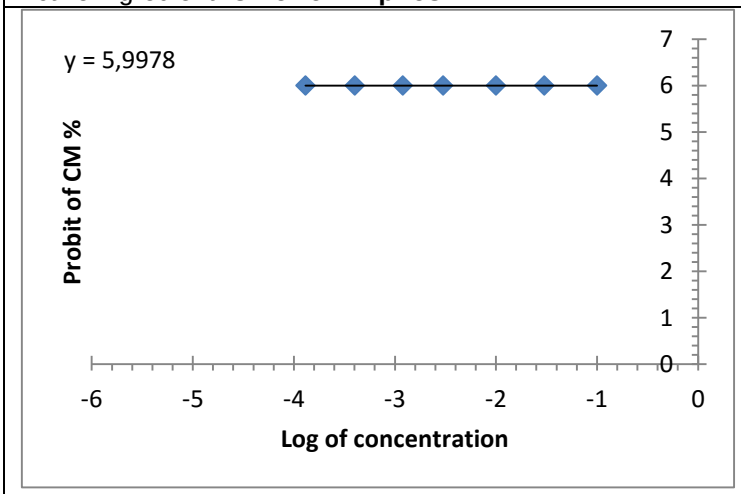


Equation y = ax + b	
a	**b**
0,826928908	7,140711522

LC	
LC50	**LC99**
2,31623 x10^{-9}	1,50668 x10^{-6}

Factor of Resistance
1,537311743

Active ingredient: **Chlorfenvinphos**



Equation y = ax + b	
a	**b**
0	5,99780702

LC	
LC50	**LC99**
Could not be determined	

Factor of Resistance
Could not be determined

Larval Immersion Test Results of *R. microplus* Ludlow population

Tick species on which tests are conducted:	*Rhipicephalus microplus*
Location on which samples were taken:	**Ludlow**
Test date: 01-12-2014	Read date: 04-12-2014

Product name: **Water control** — Active ingredient: **Water**

Water control	Test a			Duplicate test b			Calculations	
	Total	Alive	Dead	Total	Alive	Dead	M %	
	140	129	11	106	97	9	8	

Product name: **Ectodex** — Active ingredient: **Amitraz (12,5% m/v)**

Concentrations	Test a			Duplicate test b			Calculations	
	Total	Alive	Dead	Total	Alive	Dead	M %	CM %
0,000006	29	0	29	64	2	62	97,85	97,66
0,000032	11	0	11	17	0	17	100	100
0,00016	57	0	57	53	0	53	100	100
0,0008	73	0	73	55	0	55	100	100
0,004	37	0	37	55	0	55	100	100
0,02	62	0	62	56	0	56	100	100
0,1	52	0	52	37	0	37	100	100

Product name: **Ektoban** — Active ingredient: **Cypermethrin (20% m/v)**

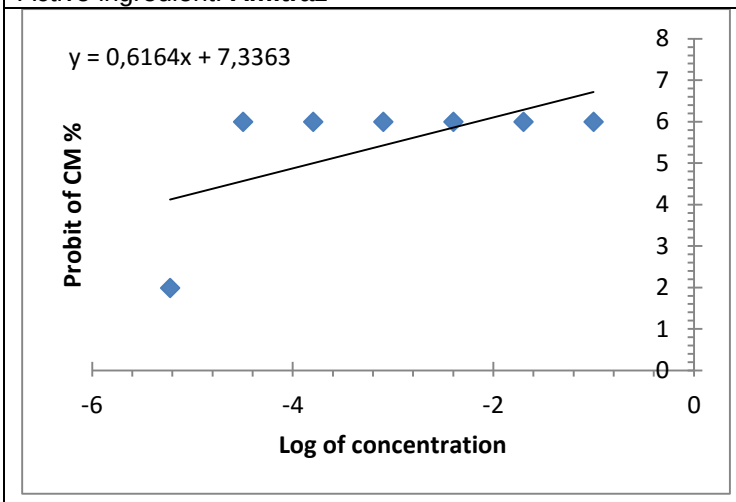
Concentrations	Test a			Duplicate test b			Calculations	
	Total	Alive	Dead	Total	Alive	Dead	M %	CM %
0,00002	31	0	31	28	0	28	100	100
0,00001	53	3	50	35	0	35	96,59	96,29
0,0005	12	2	10	10	0	10	90,91	90,10
0,002	57	0	57	15	0	15	100	100
0,01	21	0	21	27	0	27	100	100
0,05	15	0	15	17	0	17	100	100
0,2	18	0	18	21	0	21	100	100

Product name: **Supadip** — Active ingredient: **Chlorfenvinphos (30% m/v)**

Concentrations	Test a			Duplicate test b			Calculations	
	Total	Alive	Dead	Total	Alive	Dead	M %	CM %
0,00013	33	0	33	14	0	14	100	100
0,0004	19	0	19	29	0	29	100	100
0,0012	38	0	38	23	0	23	100	100
0,003	36	0	36	27	0	27	100	100
0,01	22	0	22	55	0	55	100	100
0,03	40	0	40	30	0	30	100	100
0,1	37	0	37	19	0	19	100	100

Probit CM % log concentration plots of *R. microplus* Ludlow population

Active ingredient: **Amitraz**

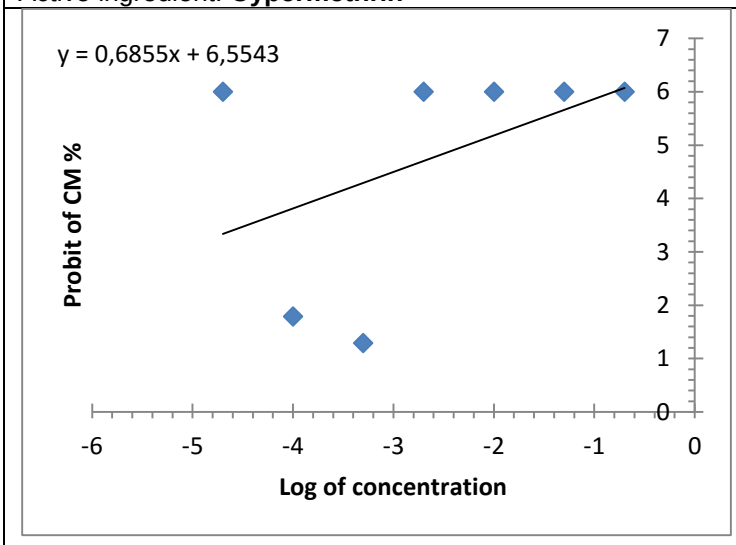


Equation y = ax + b	
a	**b**
0,616373237	7,33629104

LC	
LC50	**LC99**
1,25213 x10^{-12}	7,44561 x10^{-9}

Factor of Resistance
0,336339654

Active ingredient: **Cypermethrin**

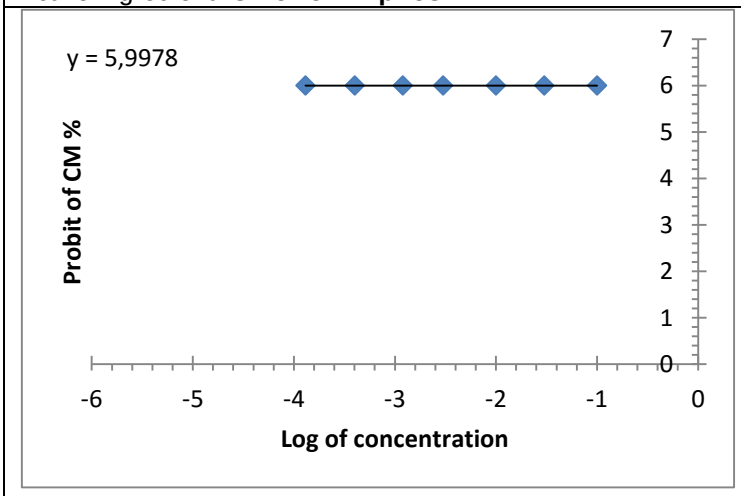


Equation y = ax + b	
a	**b**
0,685490541	6,554259696

LC	
LC50	**LC99**
2,74527 x10^{-10}	6,79646 x10^{-7}

Factor of Resistance
0,403926185

Active ingredient: **Chlorfenvinphos**



Equation y = ax + b	
a	**b**
0	5,99780702

LC	
LC50	**LC99**
Could not be determined	

Factor of Resistance
Could not be determined

Larval Immersion Test Results of *R. microplus* Tlhavekisa population

Tick species on which tests are conducted:	*Rhipicephalus microplus*
Location on which samples were taken:	**Tlhavekisa**
Test date: 01-12-2014	Read date: 04-12-2014

Product name: **Water control** — Active ingredient: **Water**

Water control	Test a			Duplicate test b			Calculations	
	Total	Alive	Dead	Total	Alive	Dead	M %	
	350	338	12	258	246	12	4	

Product name: **Ectodex** — Active ingredient: **Amitraz (12,5% m/v)**

Concentrations	Test a			Duplicate test b			Calculations	
	Total	Alive	Dead	Total	Alive	Dead	M %	CM %
0,000006	40	0	40	133	2	131	98,84	98,80
0,000032	25	0	25	49	1	48	98,65	98,59
0,00016	76	0	76	46	0	46	100	100
0,0008	31	0	31	46	0	46	100	100
0,004	38	0	38	53	0	53	100	100
0,02	34	0	34	26	0	26	100	100
0,1	61	0	61	81	0	81	100	100

Product name: **Ektoban** — Active ingredient: **Cypermethrin (20% m/v)**

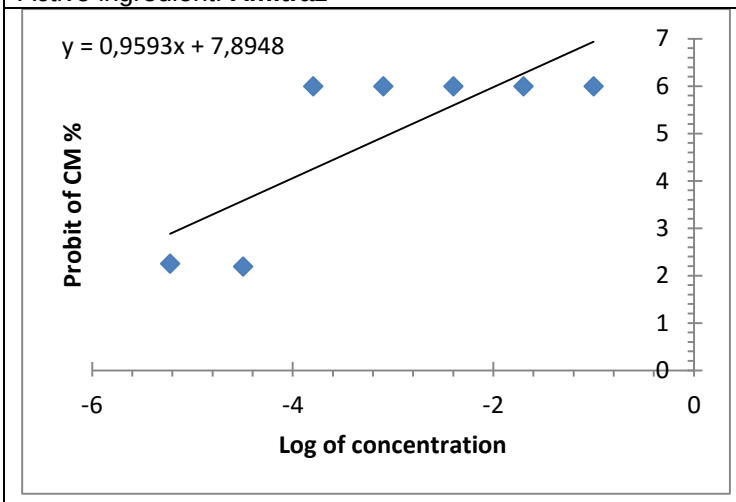
Concentrations	Test a			Duplicate test b			Calculations	
	Total	Alive	Dead	Total	Alive	Dead	M %	CM %
0,00002	58	1	57	41	0	41	98,99	98,95
0,00001	49	1	48	67	2	65	97,41	97,31
0,0005	46	0	46	22	0	22	100	100
0,002	16	0	16	18	0	18	100	100
0,01	46	0	46	98	0	98	100	100
0,05	81	0	81	77	0	77	100	100
0,2	95	0	95	128	0	128	100	100

Product name: **Supadip** — Active ingredient: **Chlorfenvinphos (30% m/v)**

Concentrations	Test a			Duplicate test b			Calculations	
	Total	Alive	Dead	Total	Alive	Dead	M %	CM %
0,00013	63	0	63	29	0	29	100	100
0,0004	43	0	43	42	0	42	100	100
0,0012	23	0	23	39	0	39	100	100
0,003	51	0	51	75	0	75	100	100
0,01	84	0	84	61	0	61	100	100
0,03	103	0	103	36	0	36	100	100
0,1	45	0	45	49	0	49	100	100

Probit CM % log concentration plots of *R. microplus* Tlhavekisa population

Active ingredient: **Amitraz**

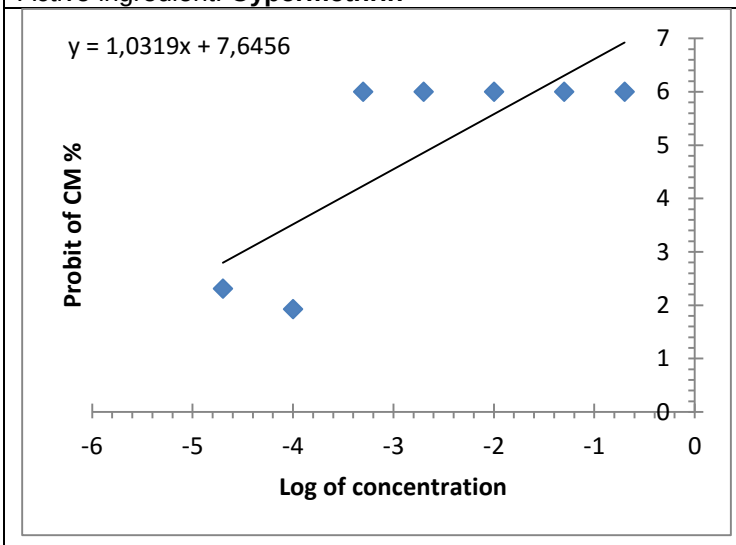


Equation y = ax + b	
a	**b**
0,959316337	7,89481196

LC	
LC50	**LC99**
5,89355 x10^{-9}	1,56813 x10^{-6}

Factor of Resistance
7,516644214

Active ingredient: **Cypermethrin**

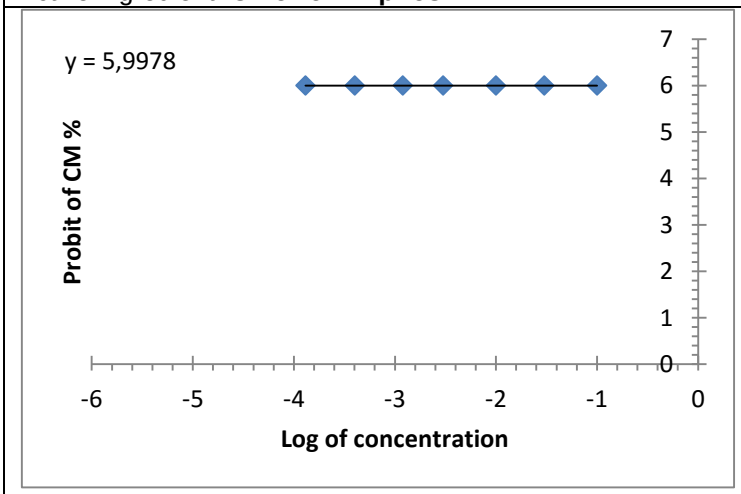


Equation y = ax + b	
a	**b**
1,031856714	7,645591569

LC	
LC50	**LC99**
3,89451 x10^{-8}	0,000007

Factor of Resistance
5,565112112

Active ingredient: **Chlorfenvinphos**



Equation y = ax + b	
a	**b**
0	5,99780702

LC	
LC50	**LC99**
Could not be determined	

Factor of Resistance
Could not be determined

Larval Immersion Test Results of *R. microplus* Utha A population

Tick species on which tests are conducted:	*Rhipicephalus microplus*
Location on which samples were taken:	**Utha A**
Test date: 05-12-2014	Read date: 08-12-2014

Product name: **Water control** — Active ingredient: **Water**

Water control	Test a			Duplicate test b			Calculations	
	Total	Alive	Dead	Total	Alive	Dead	M %	
	196	180	16	254	236	18	8	

Product name: **Ectodex** — Active ingredient: **Amitraz (12,5% m/v)**

Concentrations	Test a			Duplicate test b			Calculations	
	Total	Alive	Dead	Total	Alive	Dead	M %	CM %
0,000006	96	27	69	75	20	55	72,51	70,27
0,000032	54	8	46	65	29	36	68,91	66,37
0,00016	32	3	29	54	8	46	87,21	86,16
0,0008	167	19	148	203	2	201	94,32	93,86
0,004	24	0	24	117	0	117	100	100
0,02	36	0	36	95	0	95	100	100
0,1	124	0	124	59	0	59	100	100

Product name: **Ektoban** — Active ingredient: **Cypermethrin (20% m/v)**

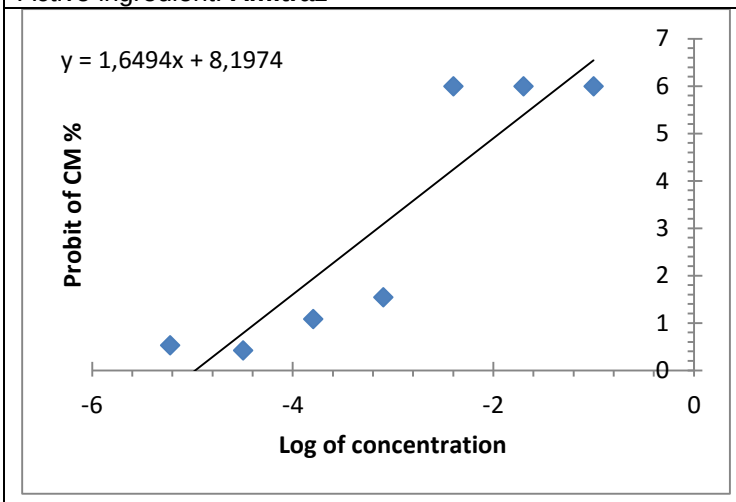
Concentrations	Test a			Duplicate test b			Calculations	
	Total	Alive	Dead	Total	Alive	Dead	M %	CM %
0,00002	158	76	82	299	156	143	49,23	45,09
0,00001	65	12	53	155	49	106	72,27	70,01
0,0005	75	11	64	101	21	80	81,82	80,33
0,002	243	37	206	93	32	61	79,46	77,79
0,01	39	2	37	91	5	86	94,62	94,18
0,05	54	4	50	23	0	23	94,81	94,38
0,2	90	4	86	50	1	49	96,43	96,14

Product name: **Supadip** — Active ingredient: **Chlorfenvinphos (30% m/v)**

Concentrations	Test a			Duplicate test b			Calculations	
	Total	Alive	Dead	Total	Alive	Dead	M %	CM %
0,00013	208	2	206	85	0	85	99,32	99,26
0,0004	116	0	116	58	0	58	100	100
0,0012	73	0	73	110	0	110	100	100
0,003	93	0	93	78	0	78	100	100
0,01	44	0	44	58	0	58	100	100
0,03	173	0	173	75	0	75	100	100
0,1	82	0	82	147	0	147	100	100

Probit CM % log concentration plots of *R. microplus* Utha A population

Active ingredient: **Amitraz**

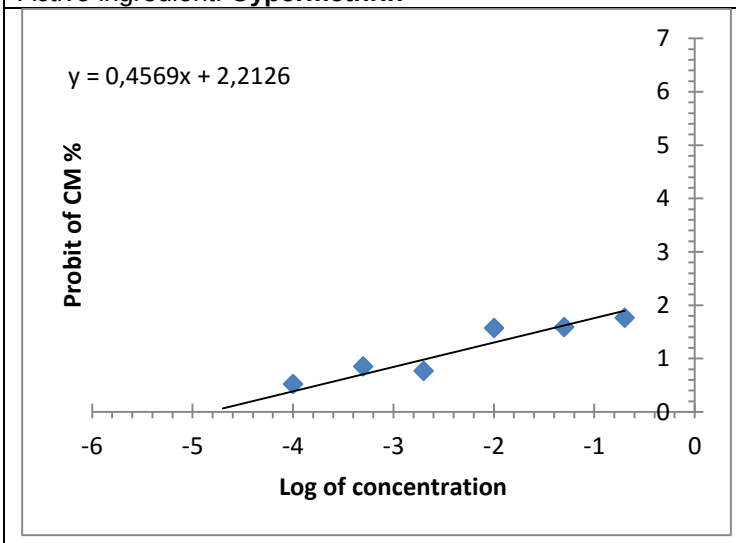


Equation y = ax + b	
a	**b**
1,649420608	8,197413175

LC	
LC50	**LC 9**
0,000011	0,00028

Factor of Resistance
77,73684936

Active ingredient: **Cypermethrin**

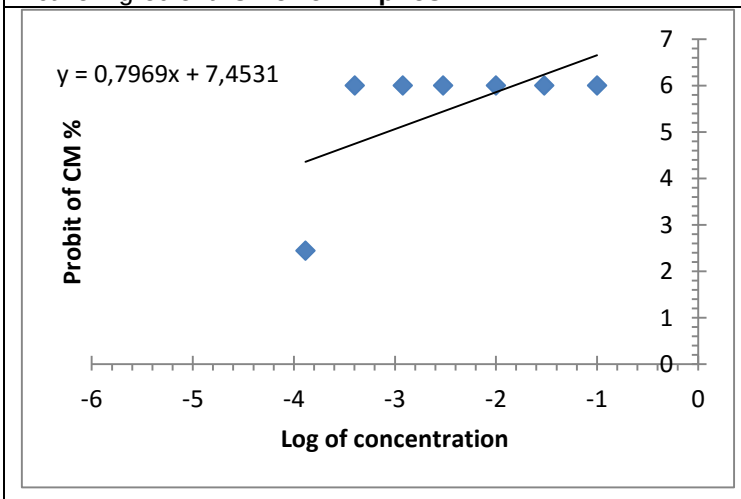


Equation y = ax + b	
a	**b**
0,456940685	2,212558233

LC	
LC50	**LC99**
0,000014	1,77429

Factor of Resistance
0,008107043

Active ingredient: **Chlorfenvinphos**



Equation y = ax + b	
a	**b**
0,79687422	7,453058046

LC	
LC50	**LC99**
4,43745 x10^{-10}	0,0000004

Factor of Resistance
0,344025

Larval Immersion Test Results of *A. hebraeum* Utha A population

Tick species on which tests are conducted:	*Amblyomma hebraeum*
Location on which samples were taken:	**Utha A**
Test date: 15-12-2014	Read date: 18-12-2014

Product name: **Water control** — Active ingredient: **Water**

Water control	Test a			Duplicate test b			Calculations	
	Total	Alive	Dead	Total	Alive	Dead	M %	
	48	44	4	49	45	4	8	

Product name: **Ectodex** — Active ingredient: **Amitraz (12,5% m/v)**

Concentrations	Test a			Duplicate test b			Calculations	
	Total	Alive	Dead	Total	Alive	Dead	M %	CM %
0,000006	106	0	106	112	0	112	100	100
0,000032	80	0	80	86	0	86	100	100
0,00016	74	0	74	90	0	90	100	100
0,0008	82	0	82	73	0	73	100	100
0,004	84	0	84	114	0	114	100	100
0,02	124	0	124	120	0	120	100	100
0,1	83	0	83	101	0	101	100	100

Product name: **Ektoban** — Active ingredient: **Cypermethrin (20% m/v)**

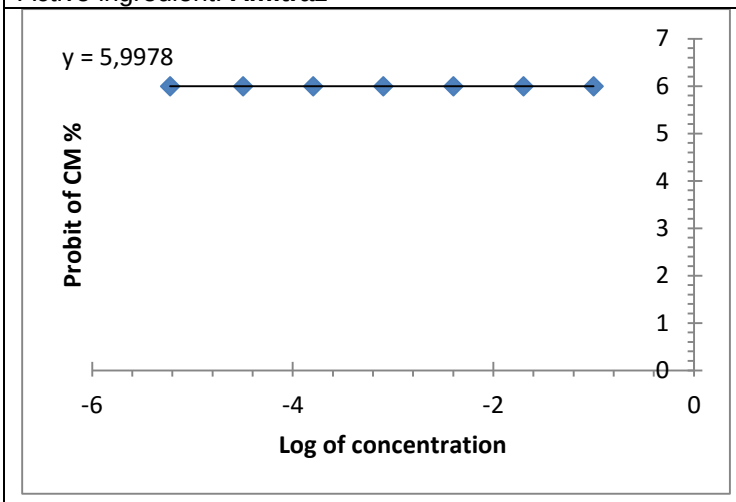
Concentrations	Test a			Duplicate test b			Calculations	
	Total	Alive	Dead	Total	Alive	Dead	M %	CM %
0,00002	102	0	102	124	0	124	100	100
0,00001	82	0	82	103	1	102	99,49	99,44
0,0005	94	0	94	102	0	102	100	100
0,002	92	0	92	82	0	82	100	100
0,01	96	0	96	94	0	94	100	100
0,05	102	0	102	92	0	92	100	100
0,2	82	0	82	96	0	96	100	100

Product name: **Supadip** — Active ingredient: **Chlorfenvinphos (30% m/v)**

Concentrations	Test a			Duplicate test b			Calculations	
	Total	Alive	Dead	Total	Alive	Dead	M %	CM %
0,00013	120	0	120	102	0	102	100	100
0,0004	128	0	128	102	0	102	100	100
0,0012	82	0	82	82	0	82	100	100
0,003	92	0	92	82	0	82	100	100
0,01	86	0	86	106	0	106	100	100
0,03	134	0	134	116	0	116	100	100
0,1	134	0	134	132	0	132	100	100

Probit CM % log concentration plots of *A. hebraeum* Utha A population

Active ingredient: **Amitraz**

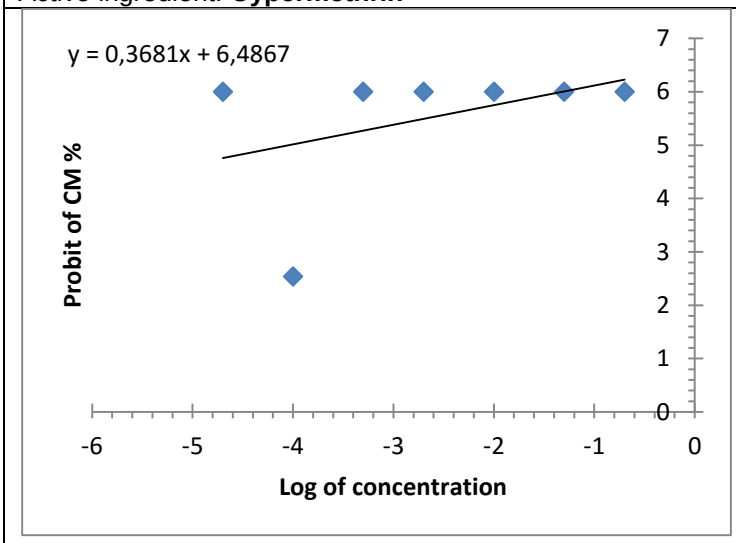


Equation y = ax + b	
a	**b**
0	5,99780702

LC	
LC50	**LC99**
Could not be determined	

Factor of Resistance
Could not be determined

Active ingredient: **Cypermethrin**

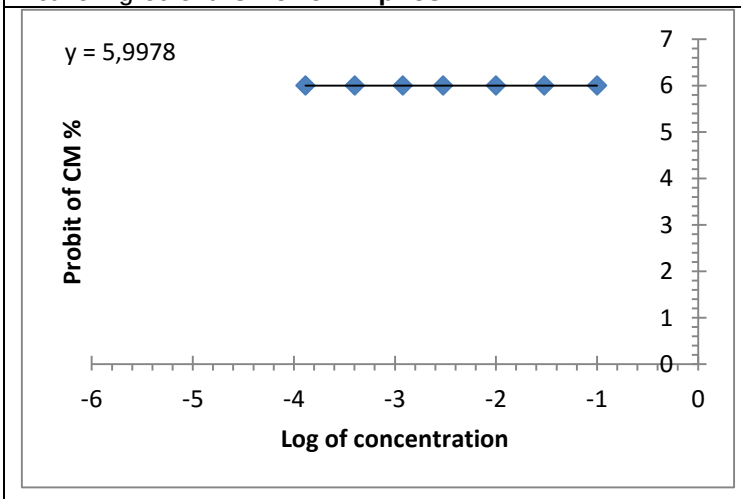


Equation y = ax + b	
a	**b**
0,368085604	6,486676816

LC	
LC50	**LC99**
$2{,}38375 \times 10^{-18}$	$4{,}98181 \times 10^{-12}$

Factor of Resistance
0,000414691

Active ingredient: **Chlorfenvinphos**



Equation y = ax + b	
a	**b**
0	5,99780702

LC	
LC50	**LC99**
Could not be determined	

Factor of Resistance
Could not be determined

Appendix D

0-100% Mortality Range Larval Immersion Test Results of *R. microplus* Utha A population

Tick species on which tests are conducted:	*Rhipicephalus microplus*
Location on which samples were taken:	**Utha A**
Test date: 15-12-2014	Read date: 18-12-2014

Product name: **Water control**					Active ingredient: **Water**				
Water control	**Test a**			**Duplicate test b**			**Calculations**		
	Total	**Alive**	**Dead**	**Total**	**Alive**	**Dead**	**M %**	
	48	44	4	49	45	4	8	

Product name: **Ektoban**					Active ingredient: **Cypermethrin (20% m/v)**				
Concentrations	**Test a**			**Duplicate test b**			**Calculations**	
	Total	**Alive**	**Dead**	**Total**	**Alive**	**Dead**	**M %**	**CM %**
0,000000000000000000262144	217	132	85	259	158	101	39,08	36,00
0,000000000000000000131072	72	44	28	77	47	30	38,93	35,84
0,000000000000000000065536	85	45	40	49	25	24	47,76	45,13
0,000000000000000000032768	59	28	31	57	27	30	52,59	50,19
0,000000000000000000016384	64	34	30	65	35	30	46,51	43,81
0,000000000000000008192	32	22	10	136	8	128	82,14	81,24
0,000000000000000004096	49	1	48	143	23	120	87,50	86,87
0,000000000000002048	46	24	22	58	28	30	50,00	47,48
0,000000000001024	51	41	10	81	40	41	38,64	35,54
0,000000000000512	33	14	19	33	15	18	56,06	53,84
0,000000000256	120	40	80	129	45	84	65,86	64,14
0,00000000128	39	1	38	40	15	25	79,75	78,72
0,0000000064	92	32	60	58	22	36	64,00	62,18
0,000000032	40	4	36	50	14	36	80,00	78,99
0,00000016	44	14	30	63	13	50	74,77	73,49
0,0000008	41	6	35	134	4	130	94,29	94,00
0,000004	44	4	40	65	3	62	93,58	93,25
0,00002	66	22	44	72	38	34	56,52	54,33
0,0001	50	10	40	21	1	20	84,51	83,73
0,0005	31	6	25	62	23	39	68,82	67,24
0,002	32	1	31	30	2	28	95,16	94,92
0,01	48	3	45	28	4	24	90,79	90,32
0,05	84	5	79	58	5	53	92,96	92,60
0,2	77	6	71	129	0	129	97,09	96,94
1%	206	0	206	128	0	128	100	100

0-100% Mortality Range Probit CM % log concentration plot of *R. microplus* Utha A population

Active ingredient: **Cypermethrin**