CHAPTER 3

METHOD OF INVESTIGATION

This research consists of two phases of experiments. The first phase was involved niche study of *Micronecta polhemusi Nieser 2000* (*MpN2000*). The niche is the set of biotic and abiotic conditions in which *MpN2000* is able to persist and maintain stable population sizes. The second experiment was involved an application of such condition for mosquito control in residential areas.

3.1 The Niche of *MpN2000*

Ecosystem or ecological system is a term first used by Tansley (1923) to describe the interdependence of species in the living world (biotic) with one another and with their non-living (abiotic) environment. The niche involves the specific area where an organism inhabits, the role or function of an organism in the ecosystem and the interrelationship of a species with all the biotic and abiotic factors affecting it. *MpN2000* niche is an aquatic ecosystem. Two man-made containers containing stagnant rain water to about three quarter full that were naturally established with *MpN2000* were examined in terms of its physico-chemical environment and the population density. The containers were an earth jar of 20 cm diameter and 30 cm height located in a housing area and an evaporation tank of 120 cm diameter and 45 cm height located in meteorological station of Universiti Teknologi PETRONAS. *MpN2000* specimens were collected from these locations using 4000 ml plastic container for detail identification of the species and its predatory behavior on mosquito larvae. The physical structures of the ecosystem and the energy input were measured. Water samples were collected for analyses on the pH, conductivity, turbidity, BOD and COD.

Figures 3.1 and 3.2 show the artificial habitat of *MpN2000* in two types of water retaining containers that established the population of *MpN2000*; Evaporation pan in meteorological station, UTP and earth jars.
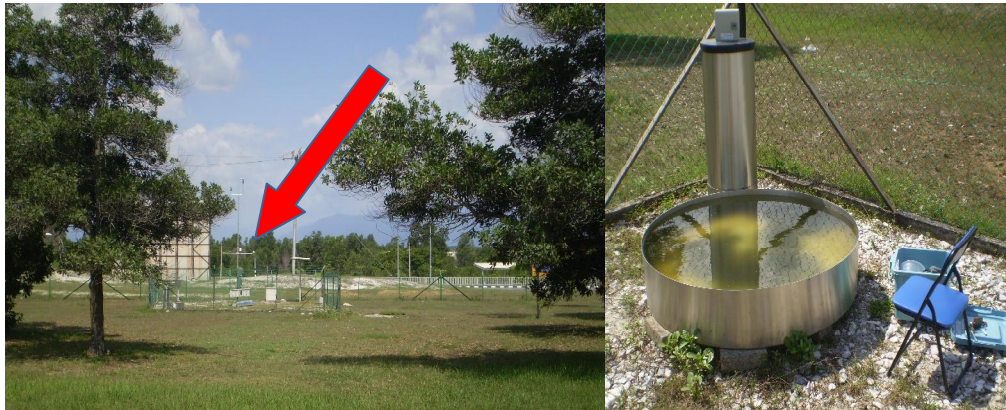

Figure 3.1 Evaporation pan, meteorological station, UTP

Figure 3.2 Earth jars contained rain water

The abiotic component identification was done on the energy (sun light intensity), water quality and humidity level as follows:

3.1.1 Analytical Measurements on Abiotic Environment

The abiotic component identification was done on the energy (sunlight intensity), water quality and humidity level as follows:

3.1.1.1 The sunlight intensity

The sunlight intensity of the ecosystems was recorded using a FP (Foto-period) meter for 12 hours on day-light for every 2 hours. The analysis were made on an averaged of 3 days records on 24th, 25th and 26th May 2008.

3.1.1.2 Water quality

The water samples were collected from the evaporation pan from meteorological station in UTP campus. Six BOD bottles were used for sampling process (Figure 3.3). The water samples were analyzed for their quality based on parameters which included BOD_5, COD, total phosphorous, ammonia nitrogen, dissolved oxygen, temperature, pH, turbidity, conductivity and light intensity.

Figure 3.3 Sampling of water from evaporation pan, meteorological station, UTP

Three water samples were sampled. The analysis was done soon as water samples were collected. The analysis of dissolved oxygen was made on site using the portable dissolved oxygen probe. Temperature and conductivity were also conducted on site. The other parameters were analyzed in the laboratory. The standard method (APHA, 1997) of analysis was applied for BOD_5.

There were also applications of different probes for pH, turbidity, conductivity, light intensity, temperature and DO for the analyses. The rest like COD, Total Phosphorous and Ammonia Nitrogen were conducted according to HACH methods (HACH, 2009). The analyses are listed in Table 3.1.

Table 3.1 The method of analysis for water quality assessment of *MpN2000* habitat water

Water Quality Parameters	Method Applied
Biochemical Oxygen Demand (BOD_5)	5-days BOD_5 Test with oxygen probe
Chemical Oxygen Demand (COD)	Chlorophosphonazo Colorimetric Method
Total Phosphorous	PhosVer® 3 with Acid Persulfate Digestion Method
Temperature	Mercury-filled Celsius Thermometer
pH	pH probe
Turbidity	Turbidity probe
Conductivity	Conductivity probe
Light intensity	FP Meter
Ammonia Nitrogen	Nessler Method
Dissolved Oxygen	Oxygen probe

3.1.1.3 Humidity level

The humidity level of the habitat at night was recorded using the humidity sensor (Testo 625 probe) over 12 hours period at night from 7pm to 5am. The data were collected over three days and each reading was made every two hours on 24th, 25th and 26th May 2008.

3.1.2 Biotic Component

The study on biotic environment of the ecosystem includes the producer (algae), the consumer *MpN2000*; the taxonomy classification, the population dynamics, its predation potential towards mosquito larvae) and the decomposer (bacteria).

3.1.2.1 Producer

The aquatic habitat for *MpN2000* that has a full exposure to 12 hour photoperiod per day was found containing algae. The analysis on the algae and other suspended solids in the water ecosystem were made based on gravimetric. The biomass of the plankton community can be estimated from gravimetric determinations, although silt and detritus may interfere (APHA, 1997).

The presence of algae in the evaporation pan of the UTP meteorological station was clearly seen from the yellowish green color of the water (Figure 3.4).

Figure 3.4 Algae as indicated by yellowish green color of the water in evaporation pan

A 1000 ml of water from *MpN2000* habitat water was filtered through 0.45 micrometer pore diameter membrane filter paper. It was cool in a desiccator and weighed. Then, the dried sample was ignited at 500 ºC for one hour. The ash was cool and re-wetted with distilled water and desiccated at 105 ºC. The ash is rewetted to restore water of hydration of clays and other minerals.

The ash-free dry weight is preferred to dry weight compare mixed assemblages. The ash content may constitute 50% or more of the dry weight in phytoplankton having inorganic structures such as diatoms. In other forms, the ash content is only 5% of dry weight.

3.1.2.2 Consumer

Three samples of 10 individual *MpN2000* each were prepared in 100 ml of rain water in universal bottles. These live samples were self transported by researcher and her supervisor during the educational trip to Universiti Teknologi Malaysia (UTM) Skudai and National University of Singapore (NUS) in 2008. These live samples were prepared as requested by the taxonomist and entomologist from Raffles Museum of Biodiversity Research, NUS, Singapore for taxonomy classification. The taxonomy identification was done within three days by the experts in the research centre. The physical structures and taxanomy of *MpN2000* were made based on the details identification the journal by Nieser, (2002). The samples were deposited in the museum samples collection for future reference. The museum has one of the largest collections of reserved insect samples in Asia (Figures 3.5 and 3.6).

Figure 3.5 The insect identification

Figure 3.6 View preservation of the insects in collection room at Raffles museum

a. The population dynamics of MpN2000

A population is a localized group of individuals of the same species. Meanwhile, population dynamics is the study of changes in population size which usually inferred from measurements of population density over time. Other attributes of populations may be important, depending on the purpose of a particular study such as distributions of size, age, sex or phenotype (e.g winged or wingless), genotype (possession of a particular sequence of DNA) or behavior (Daly *et al.,* 1998).

Experiment on population dynamic of *MpN2000* was based on distribution of size population of 30, 50 and 100 individual *MpN2000* per container, respectively. The determination of distribution size of each population was made based on EPSEM technique. EPSEM stands for Equal Probability of Selection Method. The EPSEM principle is that every element or case must have an equal probability of being selected for the sample. However, these procedures can be cumbersome when there is a long list of cases (e.g every 10th that starts with 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100) (Joseph, 2002). In this experiment, simple random sampling was practiced.

The sampling process was done to determine the average of Micronectidae per litre of water. It was 25 Micronectidae per litre of the habitat water. A capacity of 4000 ml of the habitat water was used for this experiment. Therefore, it was estimated that 4000 ml of the habitat water was convenient for 100 *MpN2000*. As for EPSEM purpose on the next stage of experiment, systematic sampling was practiced. A total of 30, 50 and 100 *MpN2000* were used for population dynamics experiment to reflect percentages of 30%, 50% and 100% of *MpN2000* population.

The experiment was conducted in control field environment using transparent plastic container of 17.5 x 25 x 12.5 cm in size seeded with these three populations of *MpN2000* (Figure 3.7).
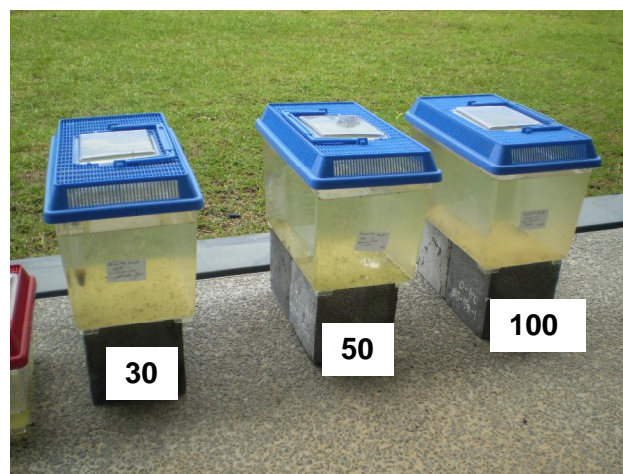

Figure 3.7 The growth pattern experiment on 3 different population densities of *MpN2000*

The plastic containers were filled with 4000 ml of the habitat water and were left under less than 12 hour photoperiod (12 hours day light) for acclimatization. The growths of the populations were counted by counting the total number of individual *MpN2000*. The growth was limited by the space and food supply as there was no additional food supply given except algae growth in the containers. Each population was observed and counted every alternate day for a total period of 40-days.

One of the earliest mathematical models of population growth was the exponential growth model based on number 1 and 2 of Figure 2.1. This model is based on the notion that the rate of change of population growth should be proportional to the population size. The larger the population, the larger the rate of growth will be. The exponential growth model equation used for this purpose is indicated in Equation 3.1 (Daly *et al.,* 1998).

$$N_t = N_0 \times e^{rt}$$
Eq.3.1

N_0 is the population size at time 0 and *e* is the base of natural logarithms with exponents *r* and *t*. N_t is the product of the growth constant and time (Daly *et al.,* 1998). This equation was used to determine the growth rate, *r* of *MpN2000* population base on its growth dynamics observation. The exponential growth model is beneficial in estimating the theoretical potential of populations to grow without restraints such as disease, competition, predation, weather or other physical limitations.

Another similar experiment was conducted in laboratory by using 64 *MpN2000*. The lab has no direct exposure to light intensity from sun and the temperature in the lab ranged from 24 to 27ºC. They were kept and observed in 30 x 60 x 30 cm glass container filled with 7000 ml of water. The growth was limited by the light intensity and temperature. Each population was observed and counted every alternate day for a total period of 30-days.

b. The predation potential of MpN2000

MpN2000 collected from the field habitats were divided into three densities group consisting of 30, 50 and 100 numbers of *MpN2000* recpectively (Figure 3.8 and Figure 3.9).
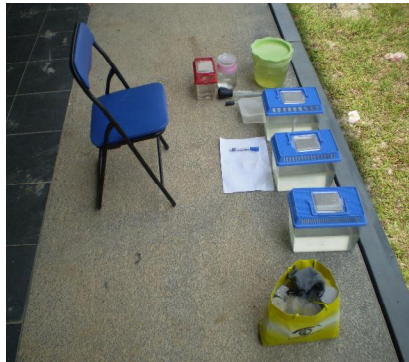

Figure 3.8 The containers

Figure 3.9 The field environment of the experiment

They were kept and observed under natural environment in artificial habitat made of 17.5 x 25 x 12.5 cm plastic containers filled with 2000 ml of the habitat water in evaporation pan, UTP. The habitat water contained of 93860 mg/L of algae mass. Each container with different population of *MpN2000* was given 50 mosquito larvae per container during the first day of the experiment. The mosquito larvae were collected particularly in discarded containers (Figure 3.10).

Figure 3.10 Mosquito breeding place where mosquito larvae were collected

Observations on the predatory pattern were made every 24 hours by counting the number of mosquito larvae left in the container. The temperature of the water in the containers ranged from 28 to 30°C with 12 hour photoperiod. Each container was fully capped with 0.2 mm air-lined plastic cover to prevent established mosquito larvae from escaping from the containers. The predation experiment was carried out for 3 days period with every day observation. There were coexistent of both algae and larvae in this experiment which reflects the food web of *MpN2000* in natural environment (Figure 3.11).
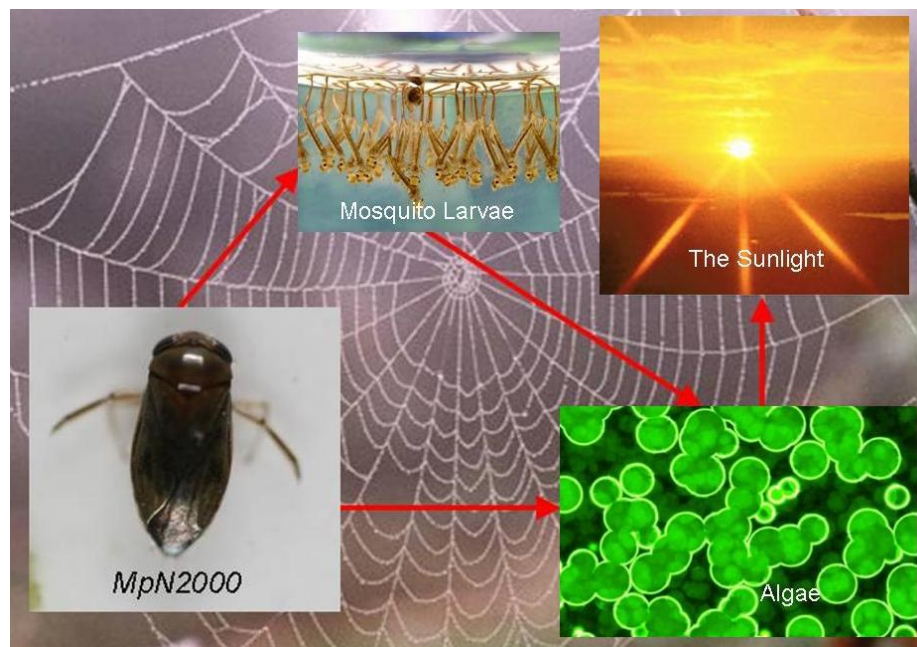

Figure 3.11 The food web of *MpN2000* in natural environment

3.1.2.3 Decomposer

Three replicates of 150 ml water samples each were sampled from evaporation pan, meteorological station, UTP to determine the bacteria population in the water body. The test was conducted by using Colilert kit for total coliform and *E.Coli* count.

3.2 The application of *MpN2000* via Model Ecosystem

Prior to the application of *MpN2000* for mosquito control, tests were conducted on the survivability of *MpN2000* under various stress conditions. There were three tests conducted; Dissolved Oxygen (DO), Biochemical Oxygen Demand (BOD_5) and temperature. For every experiment, they were tested with 4 replicates for DO, 6 replicates for BOD and 4 replicates for temperature. Each replicates was contained of certain numbers of individual *MpN2000*. The details are explained as follow.

3.2.1 The Survivability Test Procedure

The survivability test consist of three types of stressed conditions; the effect of low DO (~2 mg/L), the effect of high BOD_5 and the effect on temperature.

3.2.1.1 Effect of low dissolved oxygen (~2 mg/L)

In this experiment, *MpN2000* collected from the field habitat were divided into four population groups each consisted of 10 *MpN2000* (Figure 3.12 and Figure 3.13). These containers were sealed with aluminum foil to avoid air circulation that could increase the dissolved oxygen level. Population mortality was observed for 4 days.
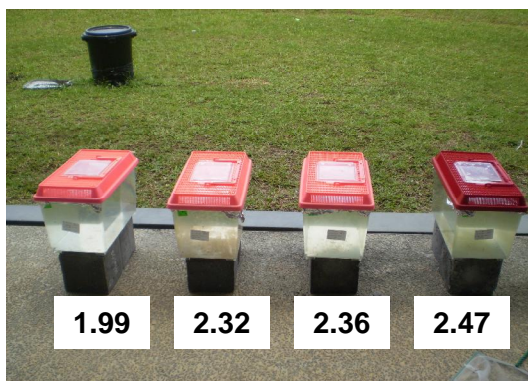

Figure 3.12 The 2 mg/L DO level

Figure 3.13 Aluminum foil was used for sealing purpose

The containers represented four different levels of dissolved oxygen that close to 2 mg/L as shown in Table 3.2.

Table 3.2 The levels of Dissolved Oxygen (DO) in the test-ecosystem

Container Numbers	DO Levels (mg/L)
1	1.99
2	2.32
3	2.36
4	2.47

3.2.1.2 Effect of organic pollutant (fish food)

Laboratory experiment on the survivability of *MpN2000* towards organic pollutant was conducted using 6 earth jars with diameter of 20 cm and 30 cm in height (capacity of 9.4 L). The jars were left in the open area adjacent to the laboratory in order to expose the jars to rain and sunlight. Each of the jars contained 7 L of rain water. Three out of six jars were seeded with 35 mg of fish food to represent polluted water and another three were kept as a control. These jars were left in control field environment. The field observations on the numbers of *MpN2000* survived and the presence of mosquito larvae in each jar were made every day for four days.

3.2.1.3 Effect of temperature

According to Subodh (2008) in his research in India, the higher temperature and humid environment are conducive for the breeding of mosquito. *MpN2000* survivability was tested in different range of temperatures to determine its tolerance. A laboratory experiments were conducted to examine the relationship between temperature and the survivability of *MpN2000* in lab environment. There were four conditions for the temperature changes; (20-22°C) BOD_5 incubator, (24-27°C) lab condition, (30-32°C) natural environment and (40-60°C) water bath. Three samples were put in every temperature indicators. These samples contained 4 individual *MpN2000* and filled with 150 ml *MpN2000* habitat water. The observations were made every day for 4 days.

3.2.2 Breeding and Acclimatization Stage of MpN2000 Population

The later phase of the study which was carried out over a period of three months focused on the application of the ecological control of stagnant water, using *MpN2000*. In this thesis, the established earth jars populated with *MpN2000* is called Model Ecosystems (ME). Ninety earth jars filled with 7L and more of rain water and seeded with 10 *MpN2000*. The jars were left under the sun to allow the *MpN2000* to multiply under natural condition. The *MpN2000* population reached in stable number after about three months.

As for preliminary step, 90 earth jars were left in open field environment for the rain water collection process (Figure 3.14). They were kept in an open area located opposite to concrete laboratory, Block 13, UTP. The observations were made in every two weeks for a month.

Figure 3.14 Establishment of *MpN2000* population in 90 earth jars

In this experiment, earth jars as man-made containers with dimension of 20 cm diameter and 30 cm in height were naturally established with *MpN2000* population. As precaution, the seeding process was done by seeding on 10 adult *MpN2000* in earth jars without *MpN2000* population. The establishment period took 3 months. It was estimated by the end of three months period, there were more than 200 individuals of *MpN2000* established in every MEs contained of rain water.

3.2.3 Introduction of Model Ecosystems (MEs) in Three Housing Areas

The test was conducted in three housing areas of Perak Tengah, Malaysia; Taman Maju (TM), Taman Tasek Putra (TTP) and Bandar Universiti (BU) as shown in Figure 3.15. The research was conducted with the collaboration from the Rukun Tetangga Committee of Taman Maju and Bandar Universiti. There was no committee for Taman Tasek Putra, therefore the approach was made by seeking permission for collaboration individually from 30 volunteers.

Figure 3.15 Location of Taman Maju (TM), Taman Tasek Putra (TTP) and Bandar Universiti (BU)
(Source: http://earth.google.com, 2009)

Table 3.3 shows the description of urban residential areas; TM, BU of Perak Tengah and TTP of Kinta Barat districts located in Perak Tengah, Perak. TM is using system of sewage treatment while BU and TTP are using centralized treatment.

Table 3.3 The the study area

Sites under Research	Ecological Description
Taman Maju (TM)	~Many part of drain clogged up with soil and ground
	~Establish vegetation found in this site
	~Sewer treatment system type: Septic tank.
Bandar Universiti (BU)	~Concrete storm-water drainage system running through the site, no stagnant water
	~Scattered vegetation among the houses, near to a stream
	~Sewage treatment system type: Septic tank
Taman Tasek Putra (TTP)	~Concrete storm-water drainage system running through the site, no stagnant water
	~Dense vegetation among the houses and surrounds by three lakes
	~Sewage treatment system type: Centralized system

3.2.3.1 Transportation

The 90 MEs were divided into three residential areas namely Taman Maju (TM), Taman Tasek Putra (TTP) and Bandar Universiti (BU). The model ecosystems were set up in these three housing areas into locations that received at least 6 hours sun light per day.

Each ME was provided with a control that was placed at a distance about 1.5 to 2 m from the ME. The controls were of the same earth jar containing rain water but without Micronectidae. All the 180 earth jars were covered with aluminum nets, labeled and acclimatized for 5 days (Figure 3.16).

Figure 3.16 The 90 MEs were covered with aluminum net before being transferred

3.2.3.2 Observation

The observations on these MEs were made every 4 days for a period of three months. The period of 4 days was chosen in order to decrease the risk of mosquito larvae evolved to be an adult mosquito. The observations were made on these four indicators listed in the analysis method below. The presence of Mosquito Larvae in ME and Control was observed and counted. The presence of *MpN2000* and other predators were recorded as biological observations only.

3.2.3.3 Analysis method

The four parameters used during the observation were;

      i. The Presence of *MpN2000* in the Controls

      ii. The Presence of mosquito larvae in the Controls

      iii. The presence of *MpN2000* in the MEs.

      iv. The presence of mosquito larvae in the MEs.

3.2.3.4 Analysis of population index

Infested Container Index (CI) (Eq.3.2) was adapted from Container Index (WHO, 1972) to estimate the mosquito population density with the presence of *MpN2000* in study area. These indices are base on the aquatic immature stages only.

The formula used for the analyses as follows:

$$ICI = \frac{Nos\ of\ Infested\ Containers}{Nos\ of\ Inspected\ Containers} \times 100 \qquad \text{Eq.3.2}$$

In this study, Micronecta Index (Eq.3.3) was formulated and calculated for comparison to Infested Container Index.

$$MI = \frac{Nos\ of\ Containers\ with\ Micronecta}{Nos\ of\ Inspected\ Containers} \times 100 \qquad \text{Eq.3.3}$$

Both indices were applied for 90 MEs and 90 controls only. The statistical analyses were done by comparing several means with two variables (ICI and MI for 21 times of observations in 3 months period) from BU and TTP (Appendix 2.1 & Appendix 2.2). Therefore, the one-way analysis of variance was carried out to find whether the observed differences between the sample means are statistically significant and thus leads to the conclusion that the corresponding population means are different. ANOVA: Single Factor was used for this purpose by using Microsoft Excel software with $\alpha = 0.05$.

T-test cannot be used as statistical analysis tool for this matter because although the sample size is between 15 and 30 (21 times of observations) and have only two variables; MI and ICI, .the population mean distribution was not reasonably symmetric and has outliers (Appendix 2.1 & Appendix 2.2). Outliers are the observations that are numerically distant from the rest of the data.

Below are the listed rules for applying T-test (Larry, 2003; Joseph, 2002; Wong & Phua, 2006):

i. If the sample size is less than 15, the population distribution should be close to normal.

ii. If the sample size is between 15 and 30, the T-test can be used when the population is reasonable symmetric and has no outliers.

iii. If the sample size is greater than 30, the T-test procedure is permissible except when there are extra outliers or strong skewess.