# EFFICACY OF GARLIC JUICE AND LEMON JUICE AS BIO-PRODUCT TREATMENTS FOR *ICHTHYOPHTHIRIUS MULTIFILIIS* ('ICH') INFECTIONS OF JUVENILE NILE TILAPIA, *OREOCHROMIS NILOTICUS*

By

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A thesis submitted to the

School of Graduate Studies

in partial fulfilment of the

requirements for the degree of

Master of Science (Aquaculture)

School of Fisheries, Marine Institute

Memorial University of Newfoundland

November, 2005

St. John's Newfoundland

*Your file Votre référence*
*ISBN: 978-0-494-19400-3*
*Our file Notre référence*
*ISBN: 978-0-494-19400-3*

**Abstract**

The ciliated protozoan, *Ichthyophthirius multifiliis,* has a worldwide distribution and almost all freshwater fish species are susceptible to its infection. Both the parasite and the resulting disease condition are popularly called ich. Currently, formalin is the only therapeutant approved for use on cultured fishes to control ectoparasites. However, the high cost and human handling safety concerns restrict the applicability of formalin for widespread use in developing countries such as Malawi. Previous studies have demonstrated garlic juice (*Allium sativum*) and lemon juice (bioflavinols) as alternative, "safe" treatments to control infestations of *I. multifiliis* and other parasites, but their efficacy has not been reported from controlled studies. To determine the efficacy of garlic and lemon juice on ich survival, two trials were conducted between July 2003 and September 2004 at the Marine Institute. Juvenile tilapia, *Oreochromis niloticus*, were infected with ich theronts and treated with different concentrations of garlic juice (1.5 – 3g/L) and lemon juice (5g/L). After infecting the fish with ich, various biological indices (condition factors, specific growth rates, feed conversion ratios, hepatosomatic indices, splenosomatic indices), blood immunology and histology samples were examined weekly for four weeks to assess treatment efficacy. Garlic juice (3g/L) in a continuous static bath exposure was the most effective treatment. Lemon juice (5g/L) was effective in killing ich theronts but resulted in unfavourable water conditions (reduced pH).

**Acknowledgements**

I would like to express my gratitude to all the people who have assisted me in completing this project and bringing me to this point in my academic and professional career.

First, I thank Dr. Duane Barker, my graduate research advisor. The completion of this project would not have occurred without his commitment and support. I also would like to thank members of my graduate committee, Dr. Laura Halfyard and Dr. Velmurugu Puvanendran for critical reviews of my thesis. The time and effort they both put into my professional growth and enlightenment will be remembered throughout my career. I would like to thank all members of staff of the Centre for Aquaculture and Seafood Development (C-ASD) and the Marine Institute International particularly Alistair Struthers, Tracy Granter, Jason Nichols, Ray Fitzgerald, Cyr Couturier, Kelly Moret, Dina Herpert, Geoff Whiteway and Echo Pittman. I also thank Dr. David Schneider for the tremendous assistance he offered in statistics and project data analysis. Special thanks to the School of Fisheries at the Marine Institute for use of the experimental facilities. I also thank Laurie Murphy, Cris Jenkins and all the friends I have known while studying here in Canada.

I give special thanks to my mom, my brothers, my sisters and all my relatives for the invaluable moral support they have accorded me during the two years of my stay in Canada.

Lastly, I thank Bill Chislett, Nigel Allen and Dr. Emmanuel Kaunda, the Bunda College/MI Project Directors, a CIDA project, for sponsoring my studies. Without this support, this project would not exist. The good LORD should bless you all.

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## List of Acronyms

Chambo: A group of tilapia species indigenous to Lake Malawi.

CF: Condition factor

FCR: Feed conversion ratio

GOM: Government of Malawi

HSI: Hepatosomatic index

'Ich': *Ichthyophthirius multifiliis* – the causative agent of the whitespot disease.

Kampango: A group of catfish (*Bagrus meridionalis*) native to Lake Malawi.

Lake Malombe: Lake fed and drained by the Shire River in southern Malawi.

LD: Lymphocyte density

ND: Neutrophil density

Shire River: The outlet of Lake Malawi.

SGR: Specific growth rate

SSI: Splenosomatic index

Theronts/tomites/swarmers: Infective stage of 'ich'

Trophozoites: Mature 'ich' on the skin, fins and gills of the fish. It is the feeding stage of the parasite commonly observed as coalescing white spots.

Usipa: A group of cyprinids, *Engraulicypris* species native to Lake Malawi.

Utaka: A group of cichlids, genus *Copadichromis* native to Lake Malawi.

## 1.0 Introduction

Aquaculture rearing systems consist of two principal types, land based and water based. The former includes ponds, raceways, tanks, and silos and the latter comprises enclosures, pens and cages. The prime function of any rearing system is to provide an environment in which stock can thrive, have adequate space and good water quality while minimising the impacts of predation, stress, disease, theft and loss through escape (Lio-Po and Lim, 2002).

Cage culture of freshwater fish, which began in Cambodia in the late 1800s, is now commonly practised in Southeast Asia and is gaining popularity in India. However, in most developing countries, this type of fish culture is still at a subsistence or semi-intensive level or is at an experimental stage (Aqua Farm News, 1993 cited in Lio-Po and Lim, 2002). In Malawi after thorough and considerable research, MALDECO Fisheries, the largest company engaged in commercial fishing in the southern part of Lake Malawi has just started cage culture. It is expected that this project will not only help boost aquaculture production in Malawi that is currently largely done by smallholder farmers using earth ponds but also supplement the dwindling catches of native chambo from Lake Malawi and other water bodies. Disease problems such as white spot disease (ich) that have been seldomly isolated in Malawi are also anticipated to increase as the level of fish culture intensity increases. According to FAO, global production from capture fisheries and aquaculture and the food fish supply is currently the highest on record and remains very significant for global food security, providing more than 15% of the total animal protein supplies (FAO, 2004). As most of the developing tropical countries use fish

ponds, their importance in complimenting the capture fisheries cannot be over emphasised.

## 1.1 Types of Ponds

Ponds used in aquaculture are classified along several lines, such as construction method, hydrology, species farmed and intensity of aquaculture. With respect to construction methods, ponds are classified as watershed, excavated and levee ponds (Egna *et al.*, 1997). Watershed ponds are formed by building a dam across a natural watercourse where topography permits water storage behind the dam. The dam is usually constructed between the two hills that constrict the watershed. Watershed ponds may store only overland flow, or they may receive some combination of overland flow, stream flow, and groundwater inflow. Excavated ponds are formed by digging a hole in the ground. They may be filled by groundwater inflow where the water table is near the land surface, by overland flow if constructed in a low-lying area, or by well water. The water in levee ponds is impounded in an area surrounded by levees. Little runoff enters levee ponds so they must be filled by water from wells, storage reservoirs, streams or estuaries. Hydrologically, ponds may be classified as static with little water exchange or as flow through where water exchange occurs regularly. From the perspective of pond dynamics, ponds are classified based on the intensity of management and the amount of production; from this, we have extensive, semi-intensive and intensive culture (Egna *et al.*, 1997). In extensive culture, there are few external inputs of nutrients, and production is low. Production is entirely dependent on the availability of natural food in the pond. Production is enhanced by application of manures or chemical fertilisers (mostly done in

earthen ponds). In semi-intensive culture systems, there are greater nutrient inputs, thus the greater production. Feeds are used to increase production along with using manure and chemical fertilisers. Intensive aquaculture systems require the highest nutrient input to achieve very high production. Large amounts of feed are applied with manure and chemical fertilisers are also often used. In addition, the ponds may be mechanically aerated.

## 1.2 Malawi

### *1.2.1 Geographic Location*

Malawi is a landlocked country located in Eastern Central Africa, bordered by Zambia in the west, Mozambique in the southwest and Tanzania in the north (Latitude 32° 40'- 35° 50' E; Longitude 9° 20' and 17° 10 S'). The total area of the country (Figure B1) is 118,500 $km^2$ of which 24% is Lake Malawi (Dickson and Brooks, 1997). Malawi has a sub-tropical climate with the rainy season (November to May) and dry season (May to November). It has a population of about 11,500,000 of which 75% live in the rural areas. Being one of the poorest countries of the world, the land holding and annual per capita income levels are estimated at as low as 2 ha and US$ 160 respectively (National Statistical Office of Malawi, 2004; State of Environment Report for Malawi, 1998).

### *1.2.2 Aquaculture Potential in Malawi*

Fish is the most important source of animal protein (60-70%) for the majority of Malawi's population. The total production and fish supply has been fluctuating between 60,000 and 80,000 tonnes for the years between 1985 and 1994, with around 85% of the

catch coming from Lake Malawi through capture fisheries (Dickson and Brooks, 1997; State of Environment Report for Malawi, 1998; Figure B2). However, fish production declined to 41,971 tonnes in 2002, with aquaculture contributing only 642 tonnes (FAO, 2004). Chambo stocks in Lake Malombe and the southeast arm of Lake Malawi have also been in serious decline in recent years (Dickson and Brooks, 1997; Figure B3). There are several reasons for the decline of chambo fishery in Malawi (Mkoka, 2003). Overfishing caused by an ever increasing number of fishers is one of the problems. The fishery is also riddled with the use of illegal gear, such as small meshed beach seine nets, that catch juvenile and immature fish. The destruction of aquatic vegetation and breeding grounds that ultimately expose the juvenile chambo to predation and fishermen's nets has also been another contributing factor. The other major problem has been the constant violation of the closed breeding season by the fishers. The end result has been illegal catching of fish during the breeding season, destroying the eggs and young fish (Mkoka, 2003).

Chambo is a general term for the three closely related species of tilapiine cichlids, presently placed by Trewavas (1982) in sub genus *Nyasalapia* of the genus *Oreochromis* (FAO, 2004). Apart from the chambo group, there are also two other species of tilapiine cichlids found in Lake Malawi: *Oreochromis shiranus* and *Tilapia rendalli*. Both are inshore species, favouring shallow swampy areas. Besides the lakes, Malawi has a number of important rivers such as the Shire that is a natural habitat for other non-tilapia species such as *Opsaridium microlepsis, O. microcephalus, Burbus eurystomus* and *B. johnstonni* (Kaunda and Chapotoka, 2003). As a result of an increase in population and

decline in total landings of fish from capture fisheries, the government is promoting aquaculture to supplement fish production in order to increase availability of fish protein to populations in upland areas and satisfy the steady growth in demand for fish (Figure B4). Currently, the aquaculture industry in Malawi is characterised by small-scale farmers and tilapia is the major fish cultured (Ambali, 2001). These farmers use earthen ponds (Figure B5) dug using either their own family labour or hired labour. Normally they use hoes and shovels during construction and use either bamboo or PVC pipes for diverting water into the ponds as inlet or outlet pipes. There are currently five commercial aquaculture farms in Malawi that are specialised in breeding and fry production. This sector has been further boosted by MALDECO Fisheries, the largest company engaged in commercial fishing in the southern part of Lake Malawi that has just started cage culture in the lake. It is anticipated that this project will greatly boost aquaculture production and positively contribute to the fragile food security problem Malawi currently faces. To protect the genetic integrity of the fish populations in the natural water bodies, the government has put a ban on importation of exotic species. Although aquaculture has developed to a considerable extent in Malawi compared to other African countries, it is still faced with the problem of culturing species with low growth rates, diseases and parasites (Ambali, 2001; Hecht and Endemann, 2004).

## 1.3 Tilapia

Tilapias (family Cichlidae) are freshwater fish that are native to Africa and the Middle East, though they have been introduced to many countries around the world (AmeriCulture, 2004; Balarin, 1979). It is the second most widely cultured fish in the

world after carp (AmeriCulture, 2004; Hrubec *et al.*, 2000; Popma and Masser, 1999). Tilapias are grouped into three genera, *Tilapia*, *Oreochromis* and *Sarotherodon* (Balarin, 1979; Popma and Masser, 1999; Pullin *et al.*, 1996). Table A1, summarises the important tilapia species and the areas commonly cultured.

(a) *Tilapia* species: are substrate spawners that guard the developing eggs and fry. They are generally herbivorous having between 7 to 16 gill rakers on the lower part of the first arch. The fry eat zooplankton while adults feed on aquatic weeds, insects, algae, and manufactured food. They generally prefer an optimum temperature of 28° C (Balarin, 1979; Trewavas, 1982).

(b) *Oreochromis* species: mouth-brood the eggs and fry. The eggs are fertilised in the nest and the females immediately pick up the eggs in their mouth and hold them through incubation and for several days after hatching. They tend to be planktivorous, having a finer set of between 10 to 28 gill rakers on the lower part of the first arch. The fry eat zooplankton while adults eat zooplankton and phytoplankton, and graze on the bottom organisms. They also eat manufactured food. They generally prefer temperatures of 25 to 30°C (Balarin, 1979; Trewavas, 1982).

(c) *Sarotherodon* species: they have similar characteristics to *Oreochromis* species. The only difference is that the males or both males and females are mouth brooders (Popma and Masser, 1999).

### *1.3.1 Tilapia Growth Phases*

Tilapia growth is divided into three phases: exponential, linear and plateau (AmeriCulture, 2004). The young fry are ravenous eaters and can consume feed that is equivalent to 25% of their body weight daily. As a result, they grow very fast when measured in percent body weight per day. This phase is referred to as exponential or accelerating. During the second phase, tilapias eat approximately the same amount each day and growth is fairly linear. Their feeding rate does not change drastically during this period because even though the fish are growing, they eat less feed as percent body weight. In the plateau phase, growth decelerates. At this point it takes more food and time to achieve a certain amount of growth (AmeriCulture, 2004).

### *1.3.2 Tilapia Diseases*

Aquaculture of tilapia as with other finfish species is adversely affected by production related disorders and infectious diseases (Hrubec *et al.*, 2000). However, tilapias tolerate adverse water quality and other stressors better than most other commercial aquaculture species (AmeriCulture, 2004; Popma and Masser, 1999). Because stress and environmental quality play an important role in the disease process, tilapias are considered to be very disease resistant. However, they still harbour many infectious and non-infectious diseases. These diseases become enzootic in intensified fish culture systems where there can often be an imbalance in the equilibrium relationship normally set up between the host and parasite. Several major contributing factors include (Balarin, 1979):

(a) The large quantity of organic materials found in ponds due to fertilisation, supplementary feeding and feces which form an ideal habitat for microorganisms.

(b) Less likelihood of the organisms being swept away in the stagnant water and the higher temperature favours rapid pathogen development.

(c) Crowded conditions which provide greater opportunities for contact between fish and there are greater encounters between the host and pathogen. The fish will also become stressed when crowded.

(d) Greater survival rates that allow less genetically fit individuals to survive. These individuals can be more susceptible to pathogens.

Consequently, bacterial, viral, fungal and parasitic diseases become problematic under such culture conditions. The most common bacterial species in tilapia are *Aeromonas liquefaciens, A. pseudomonas* and *Flexibacter columnaris*. These have been previously isolated under intensive culture systems in Kenya and Taiwan (Balarin, 1979; Tonguthai and Chinabut, 1997). Tilapias are also vulnerable to fungal infections and the common fungal species problematic are *Saprolegnia* and *Branchiomyces*. Heavy mortalities of young *S. mossambicus* due to infectious pancreatic necrosis (IPN), a virus, in cultured tilapia have also been reported in Hawaii (Balarin, 1979). Lymphocystis, a viral disease of connective tissues particularly in the dermal layer of the skin has also been isolated in intensive tilapia culture (Tonguthai and Chinabut, 1997). Most of these disease problems are not only a problem in tilapia but also common among cultured salmonids in North America, South America and Europe (Kent and Poppe, 1998; Untergasser, 1989).

Generally, parasites do not severely damage the fish unless they are present in large numbers and attack vital organs. However, blood-feeding parasites may cause severe damage even if they are only present in small numbers. Their detrimental effects are particularly severe in small fish (Tonguthai and Chinabut, 1997).

## 1.4 External Parasites

External parasites are the most frequent problem in cultured tilapia. In wild populations, external parasites are commonly found but are present in relatively low numbers on individual fish (Tonguthai and Chinabut, 1997). In pond culture, environmental conditions, such as long periods of 'drier' climate and high organic matter levels in the pond water from intensive fertilisation and feeding, provide an excellent basis for increased transmission and intensive spread of many external parasites. Because of heavy infestations, many gill, fin or skin parasites, normally known to be less harmful to fish may become pathogenic in such conditions. Further, the increased density of fish populations in the commercial ponds increases the likelihood of epidemic outbreaks (Sarig, 1985).

Many species of external parasites shed easily from the skin of fish. However, some that penetrate through the skin or have attachment organs firmly embedded into the flesh are much more difficult to dislodge. Many of these parasites may predispose the fish to secondary bacterial infection (Tonguthai and Chinabut, 1997). The most common problematic ectoparasites in tilapias include protozoa, monogenea and crustacea.

## 1.5 Protozoan Diseases

Protozoa are unicellular, microscopic organisms. They have the ability to multiply on or within a host. Not all protozoa associated with fish produce overt clinical diseases. Some are harmless commensals, whereas, others may cause clinical signs of disease only if they are present in large numbers or when the host is stressed. However, protozoa cause more disease in cultured fish than any other parasite group (Stoskopf, 1993). Harmful effects of parasitic protozoa on the host depend on their prevalence, health status of the host, site of infection and environment. Alteration of the environment may be to the advantage of the parasite. For example, intensive culture systems will favour protozoan populations (Tonguthai and Chinabut, 1997). Most external protozoa have a direct life cycle and reproduce by binary fission on the skin and gills of their hosts. Furthermore, the rate of reproduction of most protozoa depends on water quality and temperature with parasites completing their life cycles faster at higher temperatures (Tonguthai and Chinabut, 1997). Some protozoa can survive for a short time without a host, but in a confined area such as a pond, with large numbers of potential hosts, protozoa can readily spread within the fish stock.

Heavy infestations with protozoa may provoke copious mucus exudates, abnormal behaviour or coloration of the fish as well as degeneration and death of the skin epithelial cells (Tonguthai and Chinabut, 1997). Severe damage to the gill tissues not only disrupts normal function of the gills, but also predisposes the fish to secondary bacterial infection. Heavy mortalities have also been reported due to osmotic imbalance in young fry where the gills have been severely damaged (Tonguthai and Chinabut, 1997).

## 1.6 *Ichthyophthirius multifiliis*

*Ichthyophthirius multifiliis* (Fouquet) is probably the most common ectoparasite of all freshwater fishes including tilapias (Tonguthai and Chinabut, 1997). Ich has a worldwide distribution and almost all freshwater species are susceptible to its infection. It is a holotrichous, histophagous, ciliated protozoan that has a horse-shoe shaped macronucleus with at least one small round micronucleus (Dickerson and Dawe, 1995; Post, 1983; Figure 1). The micronuclei of ciliates are transcriptionally inactive and play a role in genetic exchange (Dickerson and Dawe, 1995). The mature parasite reaches approximately 1mm in diameter and is commonly observed on gills and /or skin as coalescing white spots (Figure B6). Both the parasite and the disease condition are also commonly called ich.

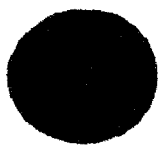

Figure 1. Wet mount of ich (40X). Source: Sustainable Fish Farm at the Earth Centre (2003).

The life cycle of this parasite is direct with some stages spent on the host and others free-swimming (Figure 2). The trophozoites mature within the host skin. The trophonts (mature trophozoite) leave the host and secrete a thick gelatinous coating and encyst. The mature trophont produces about 250 – 1000 infecting units (theronts). Once the theronts are released from the ripe trophont, they actively swim to seek the host. Those that fail to find the host within 48 hours (at 24° C - 26° C) die. The successful theront penetrates the

host skin and gill epithelium, develops into tomonts and trophozoites where they enlarge until they are visible as a white spot (Figure B6). The life cycle of ich is temperature dependent taking 3 to 4 days at 22°C, up to 11 days at 15°C and nearly 30 days at 10°C (Aquarium.net, 1997; Post, 1983; Stoskopf, 1993).

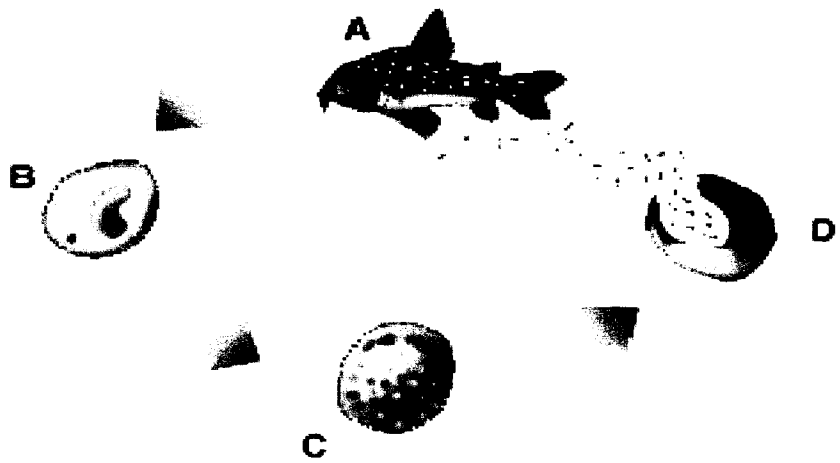


Figure 2. Life cycle of ich (The PetsForum Group, 2004). A - the trophozoites in the host's skin. B - trophont leaving the host. C - the mature trophont with hundreds of maturing theronts. D - the releasing of theronts that penetrate the skin of the host fish.

*1.6.1 Ich Pathology*

1.6.1.1 Host Behaviour Modification

In the early stages of infection, fish congregate near water intakes to reduce contact with free-swimming theronts. The fish will also 'flash' or rub their bodies against objects in reaction to skin and gill irritation caused by the theronts (Kabata, 1985; Post, 1983). As the disease progresses, the infected fish will become less active and congregate at the bottom of the pond or aquaria. The fish will also lie near the edges of the ponds and move

their gill opercula rapidly in an attempt to obtain more oxygen. With a heavy infestation, the fish will become lethargic and stop feeding (Kabata, 1985).

1.6.1.2 Gross Pathology

A heavy infection of ich results in the presence of white spots on the surface of the fish. Each spot represents the developing trophont. After the spot ruptures, releasing the mature trophont, ulcers develop on the skin making the fish more prone to secondary bacterial infection. The fins often become frayed from loss of tissue between the fin rays. Histopathology reveals tissue hyperplasia, increased mucus, cell density and tissue necrosis (Kabata, 1985; Post, 1983; Stoskopf, 1993; Tonguthai and Chinabut, 1997; Figure 3). In some instances, the parasites are localised on the gills and white spots are not seen on the skin. Affected gills are usually pale and swollen (Harper, 2003; Stoskopf, 1993).

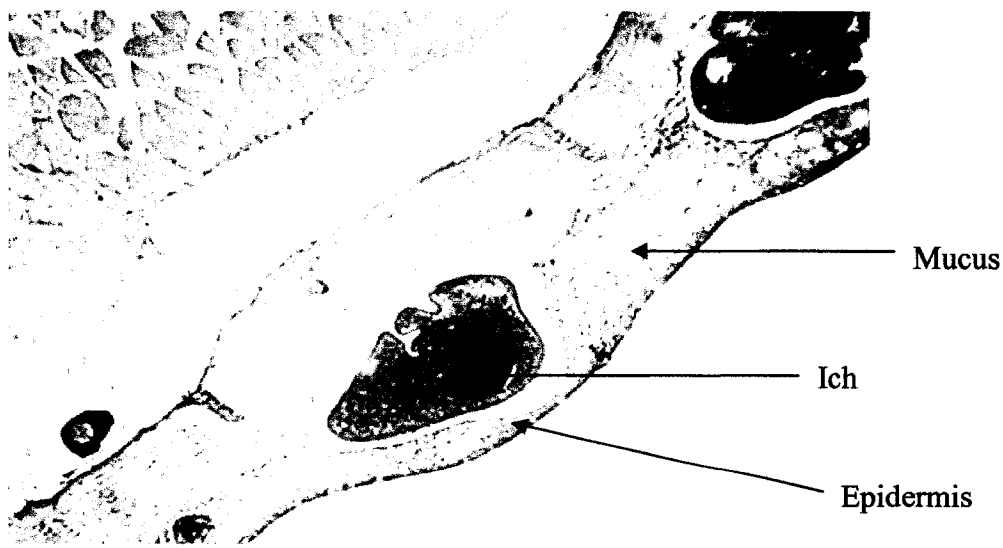

Figure 3. Trophozoites of ich in the gill tissue of the fish (Oklahoma State University Parasitology Teaching Resources).

1.6.1.3 Pathology and Virulence of Ich

Durborow *et al.* (1998) have reported the pathology of *I. multifiliis* after tissue invasion. The gill epithelium reacts to ich invasion by thickening (hyperplasia) which results in the restriction of oxygen flow. The respiratory folds of the gills (lamellae) also become deformed thereby further reducing the transfer of oxygen. The shear numbers of ich covering the gills may also cause mechanical blockage of oxygen transfer. These effects stress the fish by impairing respiration. In addition, the epithelial layer of the gills may also separate and cause loss of electrolytes, nutrients and fluids from the fish which create an osmotic imbalance. Ultimately, the damage to the gill tissue renders the fish more prone to the secondary bacterial and fungal infections. On the body of the fish, the epithelium is pushed outwards to form a white swelling as the parasite grows under the integument, which will eventually rupture, exposing the dermis to secondary infection. Heavy infestations also tend to provoke copious mucus exudates as the fish try to flush off the parasites (Tomguthai and Chinabut, 1997).

*1.6.2 Treatment of Protozoan Parasites*

Protozoan parasites are treated with various techniques:

(a) Chemicals: formalin, malachite green, bronopol, methyl blue and hydrogen peroxide used in static baths (Durborow *et al.*, 1998; Rodriguez - Tojo and Fernandez - Santamarina, 2001; Straus and Griffin, 2001).

(b) Vaccines: Free living ciliate, *Tetrahymena pyriformis* (Ellis, 1988; Houghton *et al.*, 1988).

(c) Immunostimulants: ß 1, 3 glucan products from yeast and mycelia fungi, vitamins and nucleotides. These products help boost the non-specific innate immunity of the fish (Mackie, 2003; Raa, 1996; Sakai, 1999).

(d) Natural bio-products: bioflavinols, rhubarb, garlic and lemon extracts (Buchmann *et al.*, 2003; Madsen *et al.*, 2000; Raa, 1996).

## 1.7 Purpose of the Study

Most aquaculture in Malawi is done by smallholder farmers with relatively low income (Figure B7). Chemicals such as formalin used to treat protozoan parasites and other infections are not only expensive for these farmers to use but are also associated with problems for human handling (Rodriguez - Tojo and Fernandez - Santamarina, 2001; Straus and Griffin, 2001). Furthermore, most chemicals that have proved to be effective in treating these infections, such as malachite green and copper sulphate ($CuSO_4$) have been classified as carcinogens and are banned from use on food fish (Durborow *et al.*, 1998; Ellis, 1988; Mackie, 2003; Rodriguez - Tojo and Fernandez - Santamarina, 2001; Straus and Griffin, 2001). Such factors have prompted extensive research into the use of alternative "safe" products such as immunostimulants, bioflavinols and natural bio-products (Baulny *et al.*, 1996 cited in Sealey and Gatlin, 1999, Mackie, 2003; Raa, 1996; Sakai, 1999). The present study hopes to develop a new treatment regime that can be used effectively to mitigate some of the important parasite problems in fish farms in developing countries such as Malawi.

## 1.8 Garlic and Lemon

Garlic (*Allium sativum* Linnaeus) is a perennial plant of the *Alliaceae* related to onions, chives, shallots and leeks that is mainly used as a food-flavouring agent (Lee *et al.*, 2003). Studies have shown that garlic juice has several therapeutic properties including antiparasitic, antifungal, antimicrobial, anticancer, antiatherosclerotic as well as the capacity to lower serum lipid levels (Colorni *et al.*, 1998; Miron *et al.*, 2002; Rybak *et al.*, 2004). Allicin (diallythiosulfinate) is the best known active compound of garlic that is produced by the interaction of the non-protein amino acid alliin, with the enzyme alliinase (Lee *et al.*, 2003; Figure B8). Its medicinal potential has been extensively utilised in human medicine and is currently gaining popularity in fish medicine. Allicin that represents about 70% of the overall thiosulfinates present or formed upon crushing the cloves is known to possess a vast variety of biological effects. Most of these effects are related to its strong SH-modifying and antioxidant properties (Colorni *et al.*, 1998; Miron *et al.*, 2002; Rybak *et al.*, 2004). The active ingredient in lemon extracts that has a parasiticidal effect is citronella, a terpenoid (Thomas and Callaghan, 1999). As a fish therapeutant, Madsen *et al.* (2000) reported that garlic juice completely removed *Trichodina* parasites on the eel (*Anguilla anguilla*) reared in a recirculating system. Buchmann *et al.* (2003) have also investigated the susceptibility of ich theronts and tomocysts to garlic juice. In the present study, ich infected *O. niloticus* were treated with garlic and lemon juice to evaluate comparative efficacy in treating the infection.

## 1.9 Objectives

The major objective of this research was to determine if both garlic and lemon juice have a lethal effect on theront survival. The three specific objectives were:

(a) To examine the comparative efficacy of garlic and lemon juice on ich theront survival.

(b) To determine the concentration of each product that effectively kills the ich theronts and prevents further development of the parasites.

(c) To determine the concentration of each product that can be used under practical pond culture to mitigate ichthyophthiriasis infections on tilapias.

# 2.0 Materials and Methods

## 2.1 Experimental Facility

Both trials were conducted at the aquaculture facility of the Marine Institute of Memorial University between July 2003 and September 2004. One room in the main aquaculture facility was quarantined from the rest of the system and was used as a warmwater tilapia holding room. The experimental tanks (Figure B9) consisted of 12, 54 L glass aquaria and a 164 L holding tank fitted with electric heaters and filters. All aquaria used dechlorinated freshwater and the temperature was kept constant at ~ 25° C. Juvenile tilapia, *O. niloticus* ($n$ = 1000, weight ~ 0.5 g) obtained from a commercial hatchery (*Northern Tilapia*) in Ontario on 16$^{th}$ July 2003 and 6$^{th}$ February 2004, were stocked in the holding tank for the first and second trials, respectively. These fish were fed a commercial trout starter feed (Corey Feeds) twice daily at the rate of 5% body weight per day. Standard water quality measurements were conducted every day to maintain a

suitable environment. To avoid excessive ammonia in the tank, faecal material and uneaten feed were siphoned daily and 30% of the water was changed every three days.

## 2.2 Ich and Preliminary Toxicity Trials

The original infection of ich to be used for subsequent infection trials was acquired from a local, anonymous pet store via three infected goldfish, *Carrasius auratus* and one molly, *Poecilia sphenops* (~ 6.0 g each). These fish were stocked in an 18 L holding tank on 21$^{st}$ August 2003 with an initial temperature ~ 19° C that was later changed to 23° C to increase the rate of ich multiplication and infection development. To determine what concentrations of garlic and lemon would be used in the infection trials with tilapia, a series of preliminary toxicity trials (to assess ich theront sensitivity to these products) were conducted from 26$^{th}$ September to 2$^{nd}$ October 2003. These preliminary toxicity trials were based on techniques previously described by Buchmann *et al.* (2003). However, modifications were made to the concentration of the products used and exposure times. Most of the concentrations reported by Buchmann *et al.* (2003) were not lethal to ich theronts in the present study, even after prolonged exposure (48 hours). Consequently, higher concentrations (3.0 - 8.0 g/L c.f. 0.5 – 1562.5 mg/L) and a reduced time of maximum exposure (360 min c.f. 900 min) were used in the present study compared to the ones reported by Buchmann *et al.* (2003). In the preliminary toxicity trials, garlic and lemon juice were tested in two different forms, fresh and commercially prepared. Fresh garlic was prepared at the Marine Institute from the cloves while the commercially prepared (liquid) was purchased from a local store. As part of an additional study, attempts were made to quantify the amount of allicin in the freshly prepared

crushed garlic using a High Performance Liquid Chromatography (HPLC) technique at the chemistry laboratory at the Marine Institute. However, due to uncontrollable factors little success was achieved using a technique developed by the Institute for Nutraceutical Advancement.

## 2.3 Infection Trial 1

### 2.3.1 *Stocking and Husbandry Practices*

The first infection trial was conducted between July 2003 and December 2003 using 300 fish. In trial 1, each of the 12, 54 L experimental glass aquaria (Figure B9) was filled with 35 L of water and stocked with 25 fish/aquarium on 11$^{th}$ December 2003. The fish were allowed to acclimate to the experimental aquaria for one week prior to the assignment of the treatments. The four treatments (each replicated 3x), garlic juice, lemon juice, garlic + lemon juice and the control (no therapeutants) were randomly assigned to the aquaria. During the experimental period, the fish were fed trout feed (1.0 mm) twice daily at the rate of 3% body weight per day. Standard water quality measurements were conducted every day and 20% of the water was changed every three days to avoid excessive ammonia in the system and maintain optimal conditions for the tilapia (Table A2). To prevent the aquaria filters from clogging, the foam (particulate) and charcoal inserts were cleaned every three days. In addition, the aquaria were siphoned daily to remove the uneaten feed and faecal material.

*2.3.2 Ich Infection*

The fish were infected with ich on 18th December 2003 (one week post stocking). To quantify the theront concentration (or density) introduced in each aquarium, average counts in four Petri dishes of theronts per 0.1 mL pipette were made with the aid of a dissecting microscope (40X). The number was extrapolated to a 1.0 L volume to determine the final concentrations and prepare the infection exposures. In each aquarium, 6 L of ich infected water corresponding to approximately 264,000 theronts per fish were added. The water temperature in all the aquaria was maintained at ~ 25° C to enhance ich multiplication.

*2.3.3 Preparation of Bio-product Treatments and Experimental Protocol*

Exposed fish were visually inspected every four days to monitor infection development by checking the gills and the body surfaces of randomly selected, anaesthetized (MS 222@ 200 mg/L) fish (2 per tank) for ich using a dissecting microscope (40X). Water samples were also randomly collected from the aquaria at the same intervals, and counted using the methods described above, to check the multiplication of ich theronts. Once theront multiplication and visual symptoms on the fish were detected, the garlic and lemon therapeutants were applied on 30th December 2003 (~ 2 weeks post infection).

Fresh garlic was peeled, crushed and blended. The required amount of garlic juice was weighed using a scale, mixed with water (1 L) and sieved using a gauze wire (0.292 mm diameter). The sieved garlic juice was put in a 4 L plastic beaker. Garlic juice (105g) was added to each of the 3 aquaria (35 L water volume) to obtain a therapeutic concentration of 3g/L. After addition of the garlic juice, it was observed that the pH in the aquaria fell

from 7.5 to ~ 5.6; to counteract this, sodium hydroxide (NaOH) (0.6306g) was added to each aquarium as a buffer. The addition of the NaOH immediately raised the pH back from ~ 5.6 to ~ 7.5 and no further pronounced pH drop occurred throughout the experimental period. Fresh lemons were crushed and sieved to remove the seeds. The sieved lemon juice was placed in a 4 L plastic beaker. Lemon juice (175g) was added to each of the aquaria (35 L water volume) to obtain a therapeutic concentration of 5g/L. A substantial decrease in pH (7.5 to ~ 4.6) occurred after addition of lemon juice, which required sodium hydroxide (2.7326g) as a buffer. Similarly, the NaOH immediately raised the pH back to ~ 7.5 and no further pronounced pH drop occurred throughout the experiment period. For garlic + lemon treatment, each juice was added to three aquaria to obtain a concentration of 2.0 g/L of each product. These therapeutic concentrations were chosen based on the results of toxicity trials and all bio-product juices were added to 35 L water volume per aquarium. A further three aquaria were used as the experimental controls (receiving no bio-product therapeutants). The fish in these aquaria were infected with ich as previously described and left untreated. No sodium hydroxide crystals were added to these aquaria.

*2.3.4 Sampling*

The first sampling of fish was done on 8$^{th}$ January 2004 (1 week post treatment; 3 weeks post infection). Five fish from each aquarium were randomly sampled weekly, and euthanised using MS 222 (400 mg/L). These fish were checked externally for lesions and parasites on the fins and on one first gill arch (left side) using the dissecting microscope (40X). To determine ich abundance, an infection category (Table 1) was used. For each

fish, weight ± (0.1g) and total length ± (0.1cm) were recorded. Splenomegaly and hepatomegaly are conditions whereby the spleen and the liver enlarge due to the impairment of the normal physiological processes associated with a disease condition and possibly accumulation of toxins (Woo, 2004). To check for evidence of these conditions, weights of liver and spleen from each fish were recorded (± 0.01g). Samples of the first gill arch (left side) and the lateral skin approximately 2 $cm^2$ (underneath the pectoral fin) were obtained and fixed in 10% buffered formalin. These were later processed for histology using standard embedding procedures and stained with haematoxylin and eosin (H&E) to evaluate the extent of ich induced cell pathology. Blood smears were obtained from each fish by cutting the tail fin and putting a drop of blood on a slide and making a blood smear. A peripheral blood smear from a healthy fish typically shows an assortment of erythrocytes (RBCs), lymphocytes, neutrophils (polymorphonuclear leucocytes), monocytes and thrombocytes (Yasutake and Wales, 1983). The slides were then stained using Giemsa (Dailey, 1996). Histopathological examination of gill and skin tissues was performed to check the extent of gross pathology using a compound microscope (400X). Specific growth rates (SGR), condition factors (CF), food conversion ratios (FCR), hepatosomatic and splenosomatic indices in each treatment were calculated using the appropriate formulae (Table 2). This first infection trial was terminated on 29$^{th}$ January 2004 (four weeks post exposure and six weeks post infection).

Table 1. Infection category used to determine ich abundance on tilapia.

| | |
|---|---|
| Absent | No parasites found either on the gill arch or fins |
| Light | < 5 parasites present on the gill arch or fins |
| Medium | 5-15 parasites found on either the gill arch or fins |
| Heavy | >15 parasites found on either the gill arch or fins. |
| Severe | > 50 parasites found on either the gill arch or fins |

Table 2. List of formulae used to calculate biological indices.

| **Parameter** | **Formula** |
|---|---|
| Condition factor (CF) | $(wt./L^3) * 100$ |
| Specific growth rate (SGR) (%/day) | [ln final wt. – ln initial wt.] / # of days * 100 |
| Splenosomatic index (SSI) | (wt. of spleen/wt. of fish) * 100 |
| Hepatosomatic index (HSI) | (wt.of liver/wt of fish) * 100 |
| Feed conversion ratio (FCR) | Food intake per fish/fish weight gain (g wet wt.) |

*Where:*

wt = weight (g)
L = length (cm)
ln = natural logarithm

## 2.4 Infection Trial 2

### *2.4.1 Stocking and Husbandry Practices*

The second trial was conducted between June 2004 and September 2004 using 180 fish. In trial 2, nine experimental aquaria were stocked with 20 fish/aquarium on 28$^{th}$ June 2004. The fish were allowed to acclimate to the experimental aquaria for one week prior to the assignment of the treatments. The three treatments (each replicated 3x) un-infected fish, infected (not treated) and infected treated with garlic juice (1.5g/L), were randomly assigned to the aquaria. The lower concentration of garlic juice used in this trial (1.5g/L c.f. 3.0g/L) was chosen in the interest of cost-saving, to test if a lower concentration could effectively control an ich infestation. In this second trial, the uninfected fish (a no ich, no garlic juice treatment) was assigned to assess any variations in fish biology associated with the holding system and not related to infection or treatments. During the experimental period, the fish were fed trout feed (1.0 mm) twice daily at the rate of 3% body weight per day. Standard water quality measurements (Table A3) were conducted every day and 20% of the water was changed every three days to avoid excessive ammonia build up in the system. As in infection trial 1, the foam (particulate) and charcoal inserts of aquaria filters were cleaned every three days and any remaining feed and faecal material was siphoned daily.

### *2.4.2 Source of Ich*

The ich infection used in the second trial was also obtained from a local, anonymous pet store *viz* eight infected goldfish, *Carrasius auratus* (~ 7.0 g) that were held in an 18 L

holding tank on 18th May 2004. The temperature in the holding tank was raised to 23°C to speed up infection development and ich multiplication.

*2.4.3 Ich Infection*

The fish were infected with ich on 5th July 2004 (one week post stocking). The theronts were introduced in each aquarium at the rate of ~235,000 theronts /fish. The theront concentration (or density) was determined as previously described in infection trial 1. Again, the water temperature in all the aquaria was maintained at 25°C to enhance ich multiplication.

*2.4.4 Preparation of Bio-Product Treatments and Experimental Protocol*

Exposed fish were visually inspected and water samples were checked for ich infection as previously described in infection trial 1. On July 26, 2004 (three weeks post infection) garlic juice (52.5g prepared as previously described) was applied to three aquaria (35 L water volume) to obtain a therapeutic concentration of 1.5g/L. As per infection trial 1, sodium hydroxide (0.6306g) was added to each aquarium to buffer the pH that fell from 7.5 to ~ 5.4. As in the infection trial 1, these crystals immediately raised the pH from ~ 5.4 to ~ 7.5 and no pronounced pH drop occurred throughout the experimental period. Six aquaria (three infected with ich but not treated and three not infected with ich) were used as experimental controls.

*2.4.5 Sampling*

The first sampling of fish was done on 9th August 2004 (two weeks post treatment). Five fish from each aquarium were randomly sampled weekly, and euthanised using MS 222 (400 mg/L). These fish were checked externally for lesions and parasites on the fins and first gill arch (left side) with the aid of a dissecting microscope (40X). To determine ich abundance, an infection category (Table 1) was used. Similar samples as described in infection trial 1 were collected and processed for histology from each fish. The second infection trial was terminated on September 2, 2004 (three weeks post infection).

## 2.5 Data Collection and Statistical Analysis

From the data collected in both infection trials several biological indices were calculated (Table 2). Descriptive statistics of each variable were calculated and an analysis of variance (ANOVA), using the General Linear Model (GLM), was performed. If the treatment means were significant ($P < 0.05$), Tukey's test was used to identify differences among treatments. The assumptions for independence, homogeneity and normality governing the use of the calculated $P$ – value were also evaluated using appropriate plots. (Dowdy and Wearden, 1983; Sokal and Rohlf, 1995). All statistical analyses were conducted using Minitab (version 13.1), SPSS for windows (version 11.0.0) and Microsoft Excel.

## 3.0 Results

### 3.1 Toxicity Trials

Garlic and lemon juice (in single and combined treatments) were lethal to the theronts. There was a consistent pattern of toxicity in the products tested with higher concentrations of each product quickly killing the theronts than the lower concentrations (Tables 3.0 – 3.4). For example, fresh garlic juice (3g/L) resulted in 100% mortality of the theronts in 150 minutes while a concentration of 8g/L resulted in 100% theront mortality in 60 minutes (Table 3.0). Curiously, there was a distinct difference in the strength of commercial garlic juice and fresh crushed garlic juice on theront survival. As a general pattern, relatively higher concentrations of commercial garlic juice were required to have lethal effects on the theronts than the fresh crushed garlic juice (Tables 3.0 and 3.2). For example, to achieve 100% theront mortality in one hour, a concentration of 8g/L fresh garlic was required; whereas, 28 g/L commercial garlic juice was required. Conversely, there was a slight difference in the toxicity of commercial lemon juice and fresh crushed lemon juice on the theront survival (Tables 3.1 and 3.3). A combination of fresh garlic + lemon juice required lower concentrations of each product (2g/L c.f. 3g/L and 5g/L respectively) to kill the theronts than what was used independently (Table 3.4). For infection trial 1, the lowest concentration of each product that proved lethal to theronts within 150 – 360 min was chosen, as the trial was designed to mimic a continuous, static bath exposure.

Table 3. Cumulative percent mortality of theronts (n = 1000) exposed to different concentrations of fresh crushed garlic.

| **Concentration** | **Exposure time (min)** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **g/L** | **0** | **15** | **30** | **45** | **60** | **75** | **90** | **120** | **150** |
| 3 | 0 | 0 | 0.2 | 0.5 | 20 | 75 | 98 | 99.5 | 100 |
| 4 | 0 | 0 | 0.2 | 1.0 | 30 | 95 | 100 | | |
| 5 | 0 | 0 | 0.4 | 40 | 90 | 100 | | | |
| 6 | 0 | 0.4 | 0.6 | 45 | 95 | 100 | | | |
| 7 | 0 | 0.6 | 1.0 | 50 | 99 | 100 | | | |
| 8 | 0 | 0.6 | 2.0 | 60 | 100 | | | | |

Table 3.1. Cumulative percent mortality of theronts (n = 1000) exposed to different concentrations of fresh lemon juice.

| **Concentration** | **Exposure time (min)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **g/L** | **0** | **30** | **60** | **90** | **120** | **150** | **180** | **360** |
| 5 | 5.0 | 20 | 60 | 75 | 85 | 90 | 98 | 100 |
| 6 | 5.0 | 24 | 60 | 75 | 87 | 92 | 99 | 100 |
| 7 | 10 | 60 | 90 | 98 | 99 | 100 | | |
| 8 | 85 | 95 | 98 | 99 | 100 | | | |
| 9 | 85 | 96 | 98.5 | 99 | 100 | | | |
| 10 | 95 | 98 | 99 | 100 | | | | |

Table 3.2. Cumulative percent mortality of theronts (n = 1000) exposed to different concentrations of commercial garlic juice.

| **Concentration** | **Exposure time (min)** | | | | | |
|---|---|---|---|---|---|---|
| **g/L** | **0** | **30** | **60** | **90** | **120** | **150** |
| 25 | 15 | 20 | 75 | 98 | 85 | 100 |
| 26 | 25 | 60 | 100 | | | |
| 27 | 40 | 65 | 100 | | | |
| 28 | 40 | 70 | 100 | | | |

Table 3.3. Cumulative percent mortality of theronts (n = 1000) exposed to different concentrations of commercial lemon juice.

| **Concentration** | **Exposure time (min)** | | | | | |
|---|---|---|---|---|---|---|
| **g/L** | **0** | **30** | **60** | **90** | **180** | **360** |
| 5 | 4.0 | 10 | 30 | 38 | 40 | 48 |
| 6 | 4.0 | 10 | 35 | 45 | 48 | 60 |
| 7 | 5.0 | 15 | 40 | 55 | 70 | 85 |
| 8 | 85 | 95 | 98 | 99 | 100 | |
| 9 | 80 | 95 | 97 | 99 | 100 | |
| 10 | 94 | 96 | 97 | 99 | 100 | |

Table 3.4. Cumulative percent mortality of theronts (n = 1000) exposed to different concentrations of combining fresh lemon juice and fresh crushed garlic.

| **Concentration** | **Exposure time (min)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **g/L of each** | **0** | **30** | **60** | **90** | **120** | **150** | **180** | **360** |
| 2.0 + 2.0 | 0 | 2.0 | 5.0 | 65 | 75 | 80 | 90 | 100 |
| 2.5 + 2.5 | 0 | 2.0 | 6.0 | 80 | 88 | 92 | 93 | 100 |
| 3.0 + 3.0 | 0 | 3.0 | 8.0 | 95 | 99 | 100 | | |
| 3.1 + 3.1 | 2.0 | 20 | 80 | 100 | | | | |
| 3.2 + 3.2 | 2.0 | 5.0 | 83 | 100 | | | | |
| 3.3 + 3.3 | 2.0 | 35 | 85 | 100 | | | | |

## 3.2 Infection Trial 1

### *3.2.1 Initial Stocking Data*

There were no significant differences among the mean initial weights (range: 27.84 – 29.01 g) and lengths (range: 11.37 – 12.20 cm) of the fish stocked in the experimental tanks ($P$ = 0.176, Table 4). Similarly, the mean initial stocking biomass ranged from 19.89 to 20.72 g/L (Table 4).

Table 4. Mean initial length and weight measurements (± SD) of tilapia (*O. niloticus*) used in infection trial 1 and trial 2.

| **Trial** | **Treatment** | **Mean weight (g) (±SD)** | **Mean length (cm) (± SD )** | **Stocking Density (g/L)** |
|---|---|---|---|---|
| Trial 1 | Garlic juice + parasites | 28.49 ± 4.64 | 12.00 ± 0.59 | 20.35 |
| | Lemon juice + parasites | 27.84 ± 3.73 | 11.37 ± 0.51 | 19.89 |
| | Garlic + lemon + parasites | 29.01 ± 4.69 | 12.20 ± 0.69 | 20.72 |
| | No bio-products + parasites (control) | 28.72 ± 4.66 | 12.11 ± 0.57 | 20.51 |
| Trial 2 | Garlic + parasites | 7.37 ± 2.93 | 7.45 ± 0.99 | 4.21 |
| | Uninfected fish (no parasites) | 5.56 ± 2.51 | 6.99 ± 1.1 | 3.18 |
| | Un-treated fish + parasites | 7.24 ± 2.69 | 7.72 ± 0.89 | 4.13 |

*3.2.2 Clinical Signs and Fish Behaviour*

In the infection trial 1, fish-flashing movements due to ich irritation were first noticed in all aquaria 11 days post infection. These flashing movements continued within the control (untreated) fish until the final sampling period. There were no clinically visible white spots on either the gills or fins of the fish. However, all fish that were randomly

sampled before the start of the bio-product treatments (control and treated groups) showed large numbers of theronts on the gills and fins. The infection levels of the fish among all treatments were categorised using a key (Table 1). During the entire experimental period, there was a reduction in the infection levels in the tanks that were treated with the bio-product treatments over time. This infection reduction was confirmed during each sampling time as the fish (five per tank) were randomly sampled, euthanised and checked for the presence and absence of the theronts on the fins and first gill arch (left side) with the aid of a dissecting microscope (40X). However for the control fish, the infection levels continued to progressively increase at each sampling time. This was confirmed by the presence of large numbers of theronts on the fins and gill arch of the sampled fish. In addition, the control fish produced excessive mucus on the skin and gills which was not observed among the treated fish. This trend of heavy mucus production was consistent until the final sampling. Furthermore, loss of appetite of the control fish was noted throughout this infection trial, which resulted in large amounts of uneaten feed remaining in the control aquaria.

### *3.2.3 Biological Indices*

#### 3.2.3.1 CF, SGR, FCR

The biological indices data met the three assumptions of analysis of variance (ANOVA). There were significant differences in the mean condition factors of the fish among the treatment groups ($P = 0.001$; Figure 4). The mean condition factor (CF) of the fish treated with garlic juice (1.59) was significantly higher ($P = 0.018$) than that of the control fish (1.46). The fish exposed to the bio-product therapeutants showed no significant

differences ($P = 0.70$) in the CF (Figure 4). There were no significant differences in both mean specific growth rates (SGR; %/day; Figure 5) and mean feed conversion ratio (FCR; Figure 6) of the fish among all experimental groups ($P = 0.635$ and $P = 0.745$ respectively).

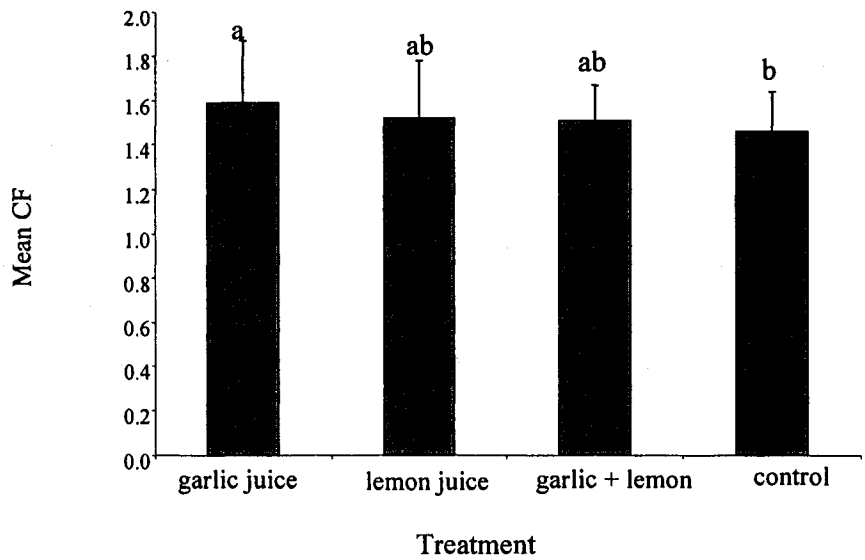


Figure 4. Mean condition factors (weight/length$^3$) * 100 of *O. niloticus* exposed to ich and subjected to four different treatment regimes (garlic juice, lemon juice, garlic plus lemon juice and control). ANOVA: N = 300, $F_{3, 240} = 2.38$. Bars represent the mean value (± SE). Different letters denote significant differences among the treatment means ($P < 0.05$).

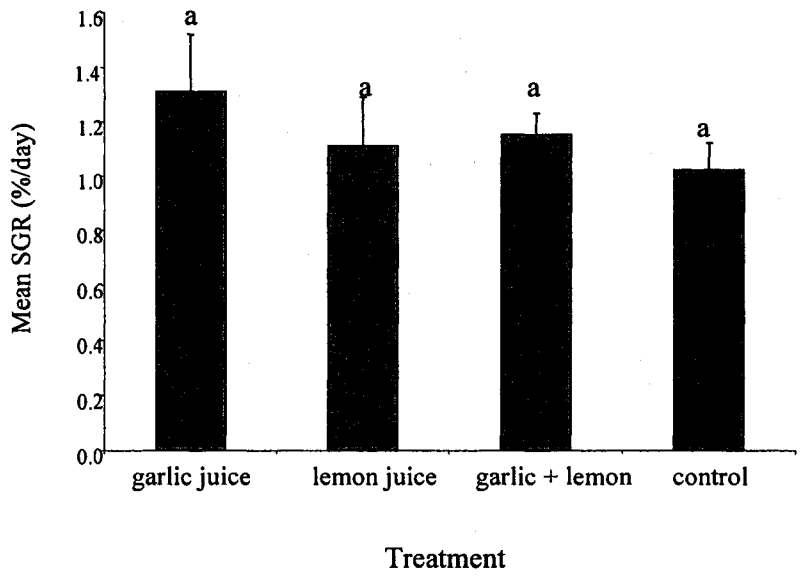


Figure 5. Mean specific growth rates (%/day) of *O. niloticus* exposed to ich and subjected to four different treatment regimes (garlic juice, lemon juice, garlic plus lemon juice and control). ANOVA: N = 300, $F_{3, 56} = 0.602$. Bars represent the mean value (± SE). Common letters denote no significant differences among the treatment means ($P > 0.05$).

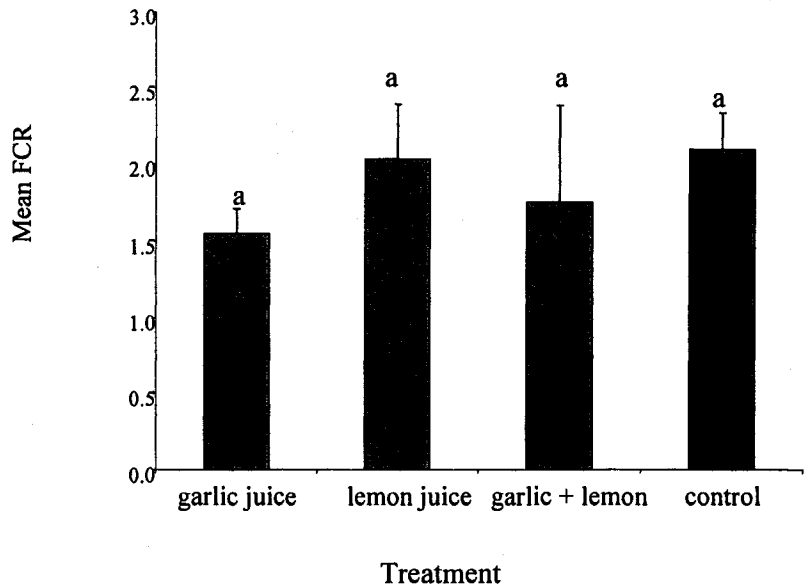


Figure 6. Mean feed conversion ratios (food intake per fish/fish weight gain) of *O. niloticus* exposed to ich and subjected to four different treatment regimes (garlic juice, lemon juice, garlic plus lemon juice and control). ANOVA: N = 300, $F_{3, 56} = 0.412$. Bars represent the mean value (± SE). Common letters denote no significant differences among the treatment means ($P > 0.05$).

3.2.3.2 Hepatosomatic (HSI) and Splenosomatic (SSI) Indices

Mean hepatosomatic (HSI) and splenosomatic (SSI) indices of the fish did not differ significantly among the treatment groups. ($P$ = 0.542 and $P$ = 0.731; Figures 7 and 8, respectively). In the infection trial 1, the mean HSI ranged from 1.73% for the fish treated with lemon juice to 2.00% for the control fish. Similarly, the mean SSI values ranged from 0.11 % for the fish treated with lemon juice to 0.12 % for the fish treated with garlic juice, the fish treated with garlic + lemon juice and the control fish (Figure 8).

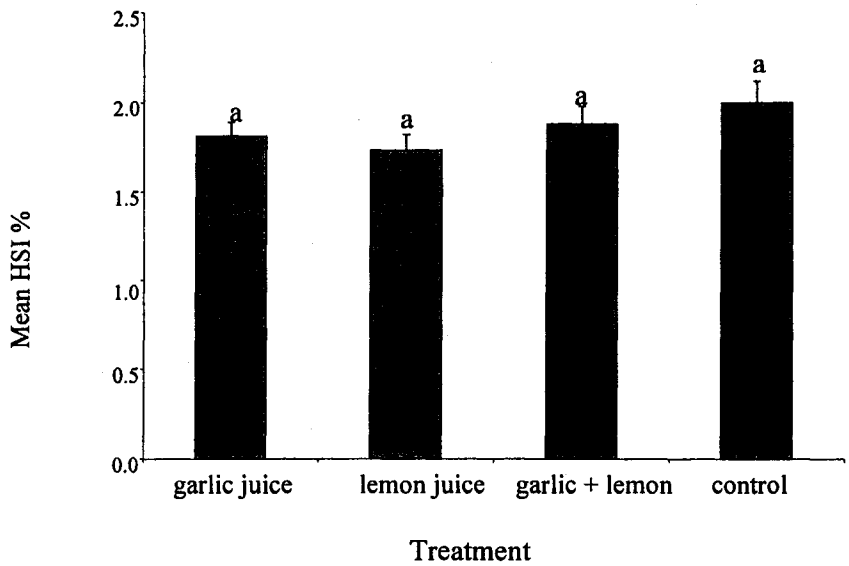


Figure 7. Mean hepatosomatic indices (weight of liver/weight of fish) * 100 of *O. niloticus* exposed to ich and subjected to four different treatment regimes (crushed garlic, lemon juice, garlic plus lemon juice and control). ANOVA: N = 300, $F_{3,\ 184}$ = 1.190. Bars represent the mean value (± SE). Common letters denote no significant differences among the treatment means ($P$ > 0.05).

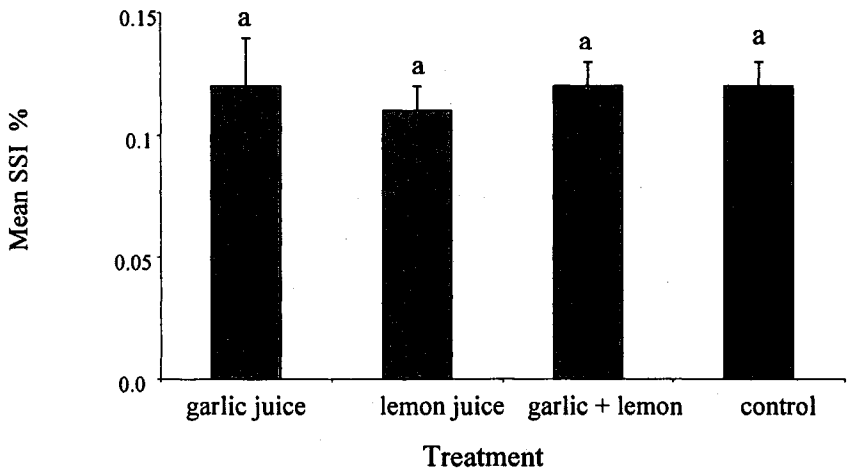


Figure 8. Mean splenosomatic indices (weight of spleen/weight of fish) * 100 of *O. niloticus* exposed to ich and subjected to four different treatment regimes (crushed garlic, lemon juice, garlic plus lemon juice and control). ANOVA: N = 300, $F_{3, 184} = 1.190$. Bars represent the mean value (± SE). Common letters denote no significant differences among the treatment means ($P > 0.05$).

3.2.3.3 Blood Immunology

There were no significant differences in the mean neutrophil densities of the fish among the treatment groups ($P = 0.147$; Figure 9). However, there were significant differences in the mean lymphocyte densities of the fish among the treatment groups ($P = 0.001$; Figure 10). In the infection trial 1, the mean neutrophil densities ranged from 3.21 for the garlic juice treatment to 5.30 neutrophils/1000 RBC's for the garlic + lemon juice treatment (Figure 9). The mean lymphocyte density of the fish treated with garlic juice was significantly higher ($P = 0.001$; 3.71 lymphocytes/100 RBC's) than the mean values of the lemon juice treated and control groups (2.66 and 1.77 lymphocytes/100 RBC's respectively). Interestingly, the control (untreated) fish had the lowest mean lymphocyte density among all experimental groups (Figure 10).

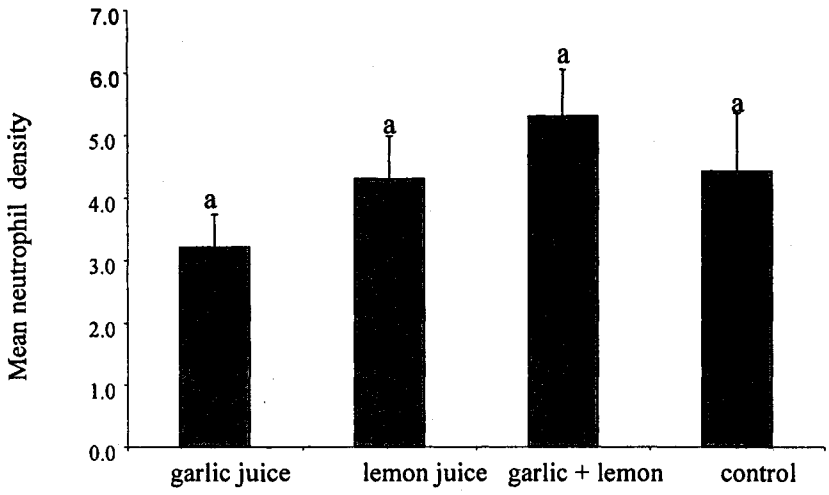


Figure 9. Mean neutrophil densities (# of neutrophils/1000 erythrocytes) of *O. niloticus* exposed to ich and subjected to four different treatment regimes (crushed garlic, lemon juice, garlic plus lemon juice and control). ANOVA: N = 300, $F_{3, 165} = 1.80$. Bars represent the mean value (± SE). Common letters denote no significant differences among the treatment means ($P > 0.05$).

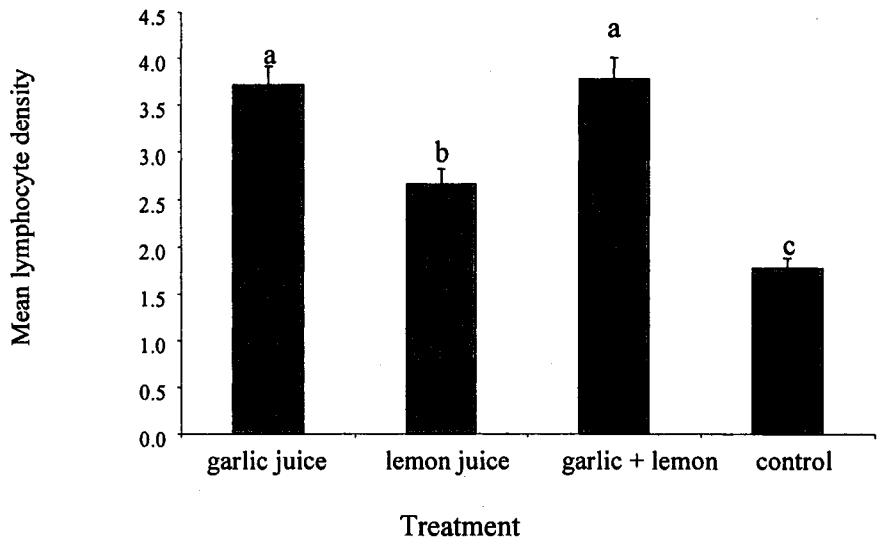


Figure 10. Mean lymphocyte densities (# of lymphocytes/100 erythrocytes) of *O. niloticus* exposed to ich and subjected to four different treatment regimes (crushed garlic, lemon juice, garlic plus lemon juice and control). ANOVA: N = 300, $F_{3, 165} = 22.43$. Bars represent the mean value (± SE). Different letters denote significant differences among the treatment means ($P < 0.05$).

3.2.3.4 Skin and Gill Histopathology

Stained sections of the skin and gills do not reveal embedded ich or the extensive cell pathology such as clubbing of the lamellae or cellular hypertrophy. However, some skin sections from the control (untreated) fish revealed a slight increase in the epidermal mucus cells compared to the treated fish.

## 3.3 Infection Trial 2

### *3.3.1 Initial Stocking Data*

There were no significant differences ($P$ = 0.153; Table 4) among both the mean initial weight (range: 5.56 – 7.37 g) and length (range: 6.99 – 7.72 cm) of the fish used in the infection trial 2. Unlike in the infection trial 1 where bigger fish were used, this trial used smaller fish. In addition, the mean initial stocking biomass ranged from 3.18 to 4.21 g/L.

### *3.3.2 Clinical Signs and Fish Behaviour*

In the infection trial 2, fish flashing and darting movements appeared occasionally at two weeks post infection. Similar to trial 1, no clinical visible white spots on either the gills or fins of the fish were observed among all experimental groups. Those fish from the ich infected + untreated aquaria exhibited a reduction in feeding which resulted in large amounts of uneaten feed accumulating on the bottom of the aquaria. In the other two experimental groups (infected + garlic treated, uninfected + untreated) no distinct change in feeding was observed.

*3.3.3 Biological Indices*

3.3.3.1 CF, SGR and FCR

Similar to infection trial 1, there were significant differences ($P$ = 0.001; Figure 11) among the mean CF of the fish from all experimental groups. The mean condition factors of the fish treated with garlic juice (1.65) and the uninfected + untreated fish (1.68) were significantly higher ($P$ = 0.019) than that from the uninfected + untreated group (1.54). However, there were no significant differences in the mean SGR and mean FCR ($P$ = 0.494 and $P$ = 0.663 respectively) among all experimental groups. The mean SGR values ranged from 1.50 %/day for the infected + untreated fish to 1.83 %/day for the uninfected + untreated fish (Figure 12). Similarly, the mean FCR values in the infection trial 2 ranged from 1.03 for the uninfected + untreated fish to 1.99 for the infected + untreated fish (Figure 13). Those fish that were infected with ich and treated with 1.5g/L garlic juice had intermediate mean SGR and FCR values.

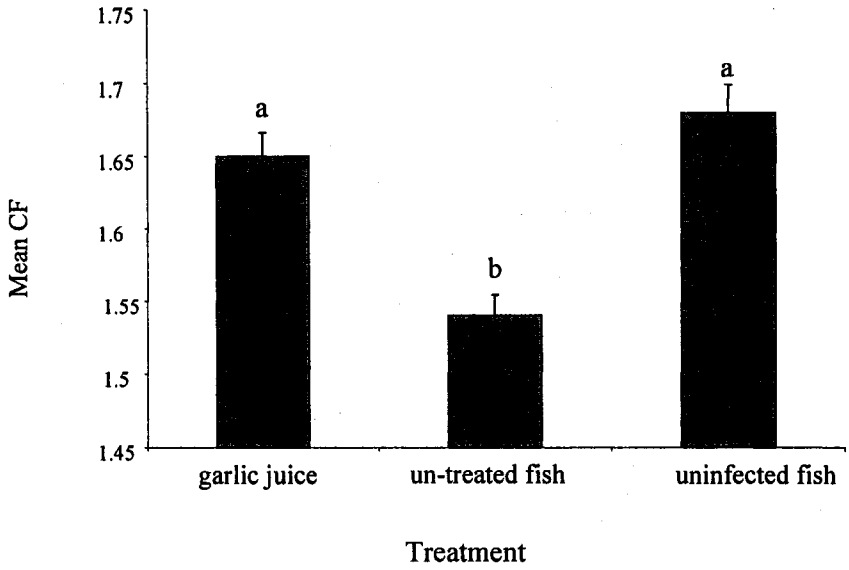


Figure 11. Mean condition factors (fish weight/fish length$^3$)* 100 of *O. niloticus* subjected to three different treatment regimes. Fish in each treatment were either infected with ich and treated with garlic juice (1.5g/L) or infected with ich and left untreated. ANOVA: N = 180, $F_{2, 42} = 14.99$. Bars represent the mean value (± SE). Different letters denote significant differences among the treatment means ($P < 0.05$).

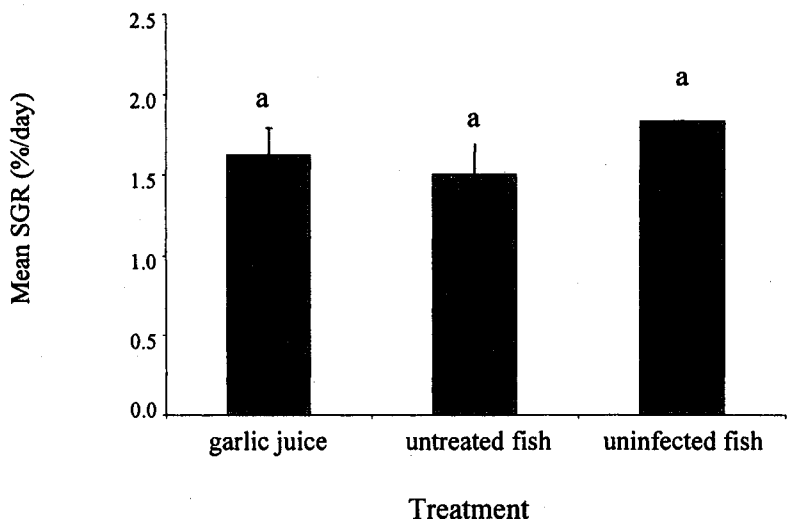


Figure 12. Mean specific growth rates (%/day) of *O. niloticus* subjected to three different treatment regimes. Fish in each treatment were either infected with ich and treated with garlic juice (1.5g/L) or infected with ich and left untreated or un-infected. ANOVA: N = 180, $F_{2, 42} = 0.72$. Bars represent the mean value (± SE). Common letters denote no significant differences among the treatment means ($P > 0.05$).

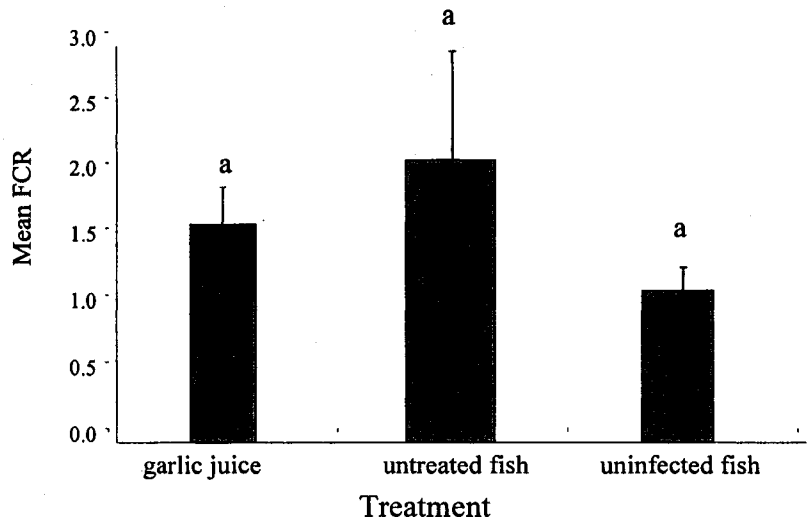


Figure 13. Mean feed conversion ratios (food intake per fish/fish weight gain) of *O. niloticus* subjected to three different treatment regimes. Fish in each treatment were either infected with ich and treated with garlic juice (1.5g/L) or infected with ich and left untreated or un-infected. ANOVA: N = 180, $F_{2, 36} = 0.42$. Bars represent the mean value (± SE). Common letters denote no significant differences among the treatment means ($P > 0.05$).

3.3.3.2 Hepatosomatic (HSI) and Splenosomatic (SSI) Indices

There were no significant differences in the mean HSI and the mean SSI among all experimental groups ($P = 0.315$ and $P = 0.886$ respectively). In the infection trial 2, the mean HSI values ranged from 2.12% for the uninfected + untreated fish to 2.40% for the infected + untreated fish (Figure 14) and the mean SSI values ranged from 0.10% for the uninfected + untreated fish to 0.13% for the infected + untreated fish (Figure 15). Similarly, those fish that were infected and treated with 1.5g/L garlic juice had intermediate mean values of both HSI and SSI.

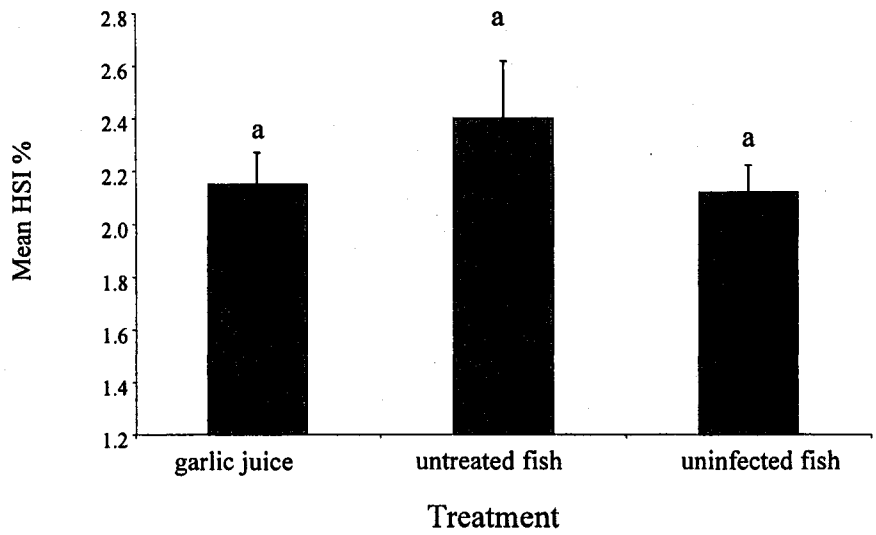


Figure 14. Mean hepatosomatic indices (weight of liver/weight of fish)* 100 of *O. niloticus* subjected to three different treatment regimes. Fish in each treatment were either infected with ich and treated with garlic juice (1.5g/L) or infected with ich and left untreated or un-infected. ANOVA: N = 180, $F_{2, 36} = 2.31$. Bars represent the mean value (± SE). Common letters denote no significant differences in the treatment means ($P > 0.05$).

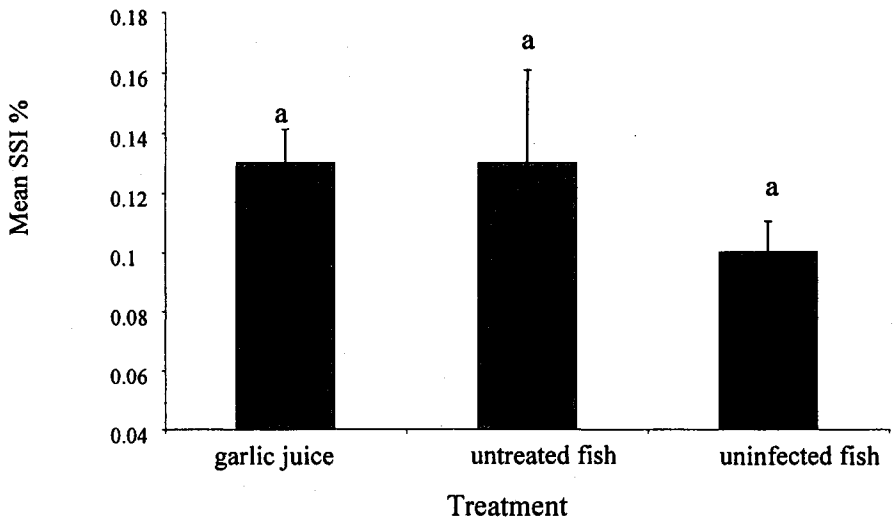


Figure 15. Mean splenosomatic indices (weight of spleen/weight of fish) *100 of *O. niloticus* subjected to three different treatment regimes. Fish in each treatment were either infected with ich and treated with garlic juice (1.5g/L) or infected with ich and left untreated or un-infected. ANOVA: N = 180, $F_{2, 69} = 2.61$. Bars represent the mean value (± SE). Common letters denote no significant differences among the treatment means ($P > 0.05$).

3.3.3.3 Blood Immunology

There were significant differences ($P$= 0.001; Figure 16) in the mean lymphocyte densities (LD) among the experimental groups. The uninfected + untreated fish had the highest ($P$ = 0.015) mean LD (4.36 lymphocytes/100 RBC's), the infected + garlic juice treated fish had the second highest ($P$ = 0.020) mean LD (3.63 lymphocytes/100 RBC's) and the infected + un-treated fish had the lowest mean LD (1.71 lymphocytes/100 RBC's; Figure 16). As with the infection trial 1, there were no significant differences ($P$ = 0.795; Figure 17) in the mean neutrophil densities (ND) among all experimental groups. The mean neutrophil density values ranged from 4.88 neutrophils/1000 RBC's for the uninfected + untreated fish to 6.08 neutrophils/1000 RBC's for the infected + untreated fish. As a general pattern in the infection trial 2, the infected + treated fish had intermediate mean neutrophil density values.

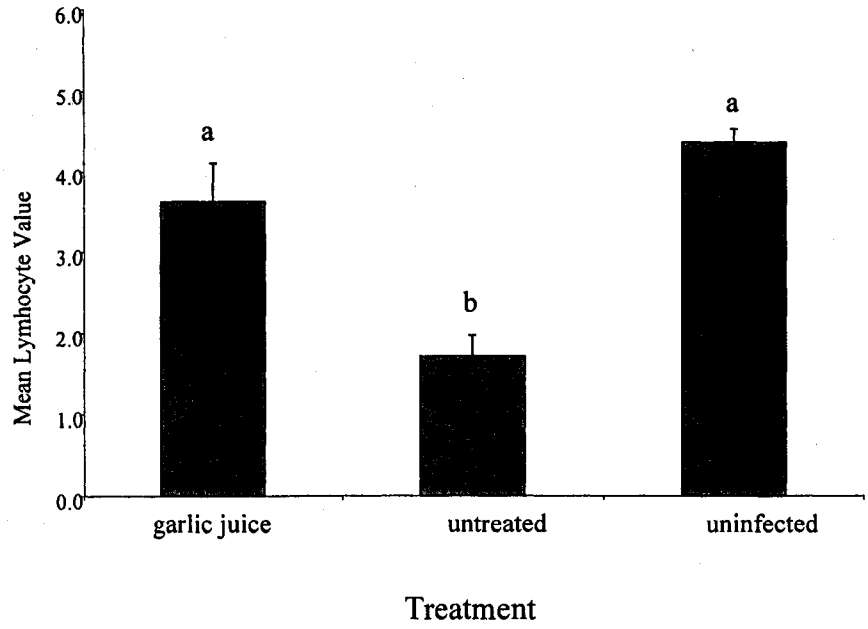


Figure 16. Mean lymphocyte densities (# of lymphocyte/100 erythrocytes) of *O. niloticus* subjected to three different treatment regimes. Fish in each treatment were either infected with ich and treated with crushed garlic (1.5g/L) or infected with ich and left untreated or un-infected. ANOVA: N = 180, $F_{2, 81} = 18.74$. Bars represent the mean value (± SE). Bars with different letters denote significant differences among the treatment means ($P < 0.05$).

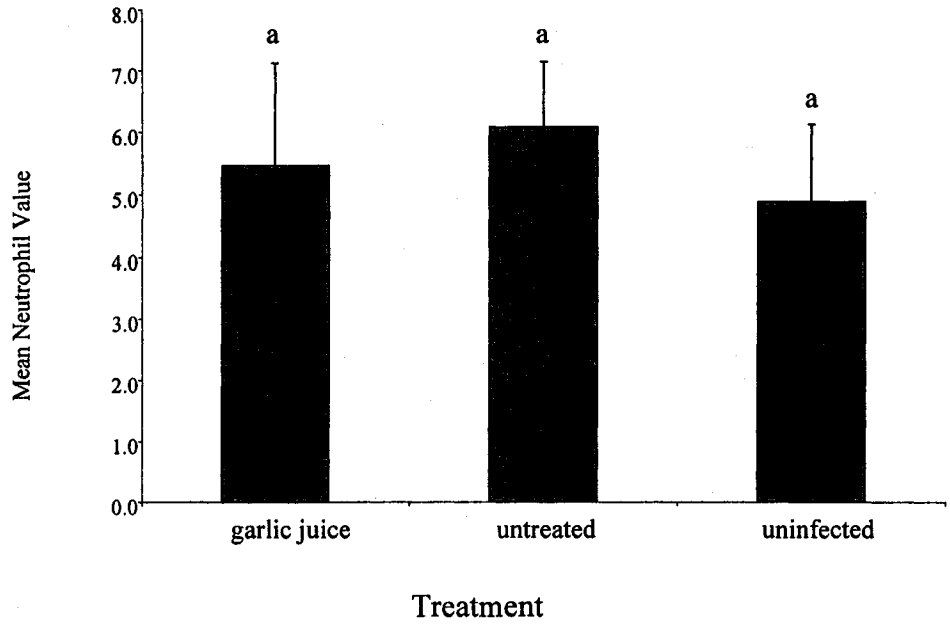


Figure 17. Mean neutrophil densities (# of neutrophils/1000 erythrocytes) of *O. niloticus* subjected to three different treatment regimes. Fish in each treatment were either infected with ich and treated with crushed garlic (1.5g/L) or infected with ich and left untreated or un-infected. Bars represent the mean value (± SE). ANOVA: $N = 180$, $F_{2, 81} = 0.230$. Bars with similar letters denote no significant differences among the treatment means ($P > 0.05$).

## 4.0 Discussion

### 4.1 Preliminary Toxicity Trials

The results of toxicity trials clearly demonstrated that garlic and lemon juice (at various concentrations) can kill ich theronts *in-vitro*. Further, higher concentrations of each product killed the theronts in a shorter time period. In a similar *in-vitro* study, Buchmann *et al.*, (2003) reported that garlic successfully killed ich theronts and tomocysts. Curiously, in the present study, much higher concentrations of garlic were required (3.0 – 8.0g/L c.f. 0.5 – 1562.5 mg/L) to effectively kill the theronts. This subsequently resulted in reduced exposure times compared to those of Buchmann *et al.* (2003). The difference in toxicity was likely associated with a difference in allicin content of garlic (associated

with different sources of origin) used in both studies. During the present study, an attempt was made to quantify the amount of allicin in the garlic juice using High Performance Liquid Chromatography (HPLC) with the assistance of the Chemistry Laboratory at the Marine Institute. Unfortunately, logistic factors (e.g. lamp compatibility, etc.) beyond my control, prevented any reliable data collection. This is obviously one area of further study.

Other studies have demonstrated a similar anti-parasitic effect of garlic in various preparations: (i) fresh raw squeezed garlic to treat *Trichodina* spp. (Ciliophora) on the eel, *Anguilla anguilla* (Madsen *et al.*, 2000) and (ii) whole garlic to control all life stages of helminth and sea lice (*Lepophtheirius, Caligus*) on salmonids (Federal Joint Subcommittee on Aquaculture, 1990). On a related note, Thomas and Callaghan (1999) reported that garlic juice and lemon peels were toxic to adult and larval stages of mosquitoes (*Culex pipiens*) with 90% of *Culex* larvae dying after only 8 hours exposure at 50ppm.

## 4.2 Infection Trials

### *4.2.1 Fish Behaviour*

In the present study, fish flashing was first noted 11- 14 days post infection. The fish that were left untreated had increased irritation of ich as evidenced by the presence of higher numbers of theronts on the gills and skin. Throughout the experimental period, these fish were flashing and also showed a general loss of appetite. These observations are consistent with other published reports of infected fish showing abnormal behaviours and

disorders associated with an infection (Post, 1983). Severe ich infections may induce anorexia in fish (loss of appetite with consequential wasting), which is followed by abnormal behaviours such as excessive hiding, rubbing and scratching ("flashing"), erratic swimming, breathing at the surface and increased ventilation rates (Aquarium.net, 1997; Kabata, 1985; Post, 1983; Untergasser, 1989). The groups that received a bio-product therapeutant exhibited no change in feeding pattern. Unlike in the fish groups that received the bio-product treatments where ich irritation (theronts) on the fish and in water kept decreasing with time, the fish that received no bio-product treatment showed a progressive increase in irritation as evidenced by flashing and ever increasing numbers of theronts on the fish and in water at each sampling period.

*4.2.2 Biological Indices (CF, SGR, FCR)*

In both infection trials, the garlic juice treated fish showed the highest mean values of CF and displayed a consistent pattern of better SGR and FCR values although differences were not statistically significant. A possible hypothesis is that garlic juice was an effective therapeutant for killing theronts, the fish resumed their normal feeding and growth was not adversely affected. In the infection trial 2, the uninfected fish displayed a similar pattern like the garlic juice treated fish in the infection trial 1 with the highest mean CF value and better SGR and FCR values (although not statistically significant) for both infection trials, time effect had no significant differences on the biological indices calculated.

Although lemon juice was lethal to the theronts *in – vitro*, it resulted in unfavourable water conditions (much reduced pH). Although I tried to buffer the water, the marked initial drop in pH may have negatively affected feeding and other physiological processes resulting in poorer growth than the garlic juice treated fish. Further, the tilapia from the lemon treatments did not display clinical symptoms of ich.

For the infected + untreated fish in both trials, the stress and irritation induced by ich likely contributed to these groups consistently displaying a pattern of low biological indices (mean SGR and FCR, although not statistically significant). These fish might have also been using much of their energy to fight the infection instead of using it for somatic growth. As noted earlier, feeding in this treatment group was adversely affected, hence the poorest biological indices. Acute disease conditions in fish may be responsible for loss of appetite, reduced feed intake and subsequent wastage of the feed through uneaten feed accumulating at the bottom of the aquaria or pond (Aquarium.net, 1997; Kabata, 1985; Post, 1983; Untergasser, 1989). Poorly feeding fish tend to lose weight, resulting in lower condition factors (Post, 1983). In addition, the fish may use energy to fight the disease that could have been used in somatic growth. Such scenarios are also closely linked to reduced SGR, and poor FCR.

*4.2.3 Hepatosomatic and Splenosomatic Indices*

The present study did not find any evidence of distinct enlargement of spleen (spenomegaly) or liver (hepatomegaly) as would be indicated by significantly higher SSI & HSI respectively. Splenomegaly, hepatomegaly, and other internal

abnormalities/pathology have been previously reported in fish with acute infections of bacteria, virus and parasites (Aquaculture Network Information Centre, 2004; Microtek International, 2004; Woo, 2004). Data from the present study imply that tilapia did not develop typical disease conditions associated with ich infection (see also section 4.2.5). However, in both infection trials, a common pattern emerged with the uninfected + untreated groups showing slightly higher SSI and HSI values (though not statistically significant). Based on several reports (Atwood *et al.*, 2003; Balarin, 1979; Popma and Masser, 1999) indicating disease resistance of cultured tilapia, a possible hypothesis is that tilapias are more disease resistant than other cultured fishes; therefore, only in severe, acute disease conditions will overt clinical symptoms be evident. Perhaps if the infections were of a longer duration or if stressful holding conditions were used, the classical symptoms would have been more pronounced.

### *4.2.4 Blood Immunology*

In both infection trials, the fish treated with garlic juice (singly or combined with lemon) showed significantly higher lymphocyte densities than those infected + untreated. A likely hypothesis is that once the ich infections and the associated stress were eliminated, the fish reverted to their normal physiological and metabolic processes. The immunology data from the uninfected + untreated fish in the infection trial 2 also support this as these fish had the highest lymphocyte density. Further, lymphocyte densities for the uninfected + untreated and those of the infected + treated fish reported in the present study fall well within the upper ranges of lymphocyte densities reported for healthy salmonids (Roberts, 1989; Yasutake and Wales, 1983). Haematology in fish, particularly the leucocyte

fraction of the blood, is influenced by several factors such as environmental poisons, lack of essential nutrients, stress, diseases and parasitic infection (Witten *et al.*, 1998). Lymphocytes are key coordinating cells of the specific immune response (memory component) in the fish and may also play an indirect role in the non-specific defence mechanisms (Ellis, 1999; Roberts, 1989).

This scenario has a direct impact on the immune capabilities of the fish. The main function of the immune system is to protect the animal against disease causing microbes. The immune system comprises both specific and non-specific components, and involves both cellular and humoral factors. Lymphocytes are the cells that are responsible for the specific immune response in the fish and also play a role in the non-specific defence mechanisms. Lymphocyte numbers tend to lower tremendously with poor environment (Dinakaran, 1997), stress (Barcellos *et al.*, 2004; Tierney *et al.*, 2004), disease (Akhlaghi and Mahjor, 2004) and parasites (Densmore *et al.*, 2004). A reduction in the number of lymphocytes ultimately indicates a reduced immune response (Ellis, 1999).

In both infection trials of the present study, there were no significant differences in the neutrophil densities. Unlike lymphocytes, neutrophils are responsible for the non-specific ('general') immune response in fish and tend to show elevated numbers (short term) at times of acute stress (Tierney *et al.*, 2004), disease onset (Ellis, 1999; Matsuyama and Iida, 1999) and injury (Roberts, 1989). The neutrophil values reported for infected + treated and the uninfected + untreated tilapia in the present study, fall within the upper range for healthy salmonids (Roberts, 1989), so there may be some indication of this

response in the tilapia. One possibility for the lack of significance would be the huge variation associated with each calculated mean neutrophil density (per 1000 RBC's). Within the same experimental group, some samples would have 1-2 neutrophils per 1000 RBC's whereas other samples displayed 'clumping' of neutrophils resulting in 10 – 15 per RBC's. Obviously a modification of the neutrophil enumeration technique is required. Besides the modification to the enumeration technique, increasing the sample number to obtain better estimates needs to be evaluated in the follow up trials.

*4.2.5 Skin and Gill Histopathology*

There was no evidence of extensive tissue histopathology in the gill or skin samples from ich infected tilapia used in the present study. However, mucus cells showed a slight elevation in some of the ich infected + untreated groups, a trend consistent with ich infections (Dickerson and Dawe, 1995). In typical ich infections on cultured salmonids and catfishes, the theronts tend to penetrate the epithelium resulting in extensive changes in the surrounding integumental tissues (Tonguthai and Chinabut, 1997). Histological gill sections cut from fish with ich will clearly demonstrate trophozoites with typical crescent – shaped/horse shoe macronucleus surrounded by an increased density of mucus cells (Dickerson and Dawe, 1995; Post, 1983; Tonguthai and Chinabut, 1997). One possible hypothesis (as previously suggested) could be high resistance to infection tilapias tend to show when infected by different kinds of diseases under varying degrees of stress (Atwood *et al.*, 2003; Tonguthai and Chinabut, 1997). Additionally, as an external 'first defence', tilapia have very thick, overlapping scales that would help fight off invading ectoparasites and other pathogens. Another possible hypothesis for the lack of extensive

cell pathology could be the relatively low stocking densities (compared with the stocking densities in the typical pond culture) that were used in the infection trials. Using the high stocking densities as are commonly used in Malawi pond culture would have been impractical for the present study (using glass aquaria) and would have resulted in increased aggression and likely, increased mortalities. Similarly, if each infection trial had been terminated later (thus having longer periods of post exposure to ich), there may have been more evidence of chronic stress among the fish. However, such an amendment in protocol would have been in violation of our Canadian Council on Animal Care (CCAC) protocols at the Marine Institute.

### *4.2.6 Practical Aspects of the Research Findings*

Both garlic and lemon juice were lethal to ich theronts. The present research findings support the results of similar work conducted by Buchmann *et al.* (2003) and Madsen *et al.* (2000). However, one key technical problem that was noted with the treatments was the reduction of the water pH. This was more pronounced with the lemon juice treatments than with garlic treatments. In the present study, the pH was effectively buffered using sodium hydroxide (NaOH) crystals that were directly applied to the treatment tanks and no further pronounced pH changes were experienced throughout the trial period. It is anticipated that under practical pond culture similar problems could arise, especially with the lemon treatment and its high acid content. This problem may easily and effectively be corrected by application of agricultural lime (calcium carbonate). Calcium carbonate ($CaCO_3$) is already used in fish ponds in Africa, Asia and other places to kill potential fish pathogens and also to raise water pH where soils are known to be acidic. Therefore

its usage to correct pH problems arising from such treatments may not pose any new technological challenge to existing farms.

## 5.0 Recommendations and Conclusions

As a caveat, there are some aspects of this research that need to be explored further before this technology is adopted on a larger scale. Firstly, conduct similar trials under pond culture to compare the results with laboratory trials. Such trials could be conducted both in concrete and earthen ponds to study treatment effects on water quality parameters (e.g. pH). This will help resolve some of the practical problems that could be associated with the treatments before the technology is taken to the farmers. Second, quantify allicin, the active ingredient compound in garlic. It would be very beneficial to have a clear indication of how much of the active ingredient is present and for what time duration. It is thought that the amount of allicin varies with strains of garlic (Lee *et al.*, 2003; Miron *et al.*, 2002), hence the need to further pursue allicin quantification. Similarly, I would recommend trying to isolate allicin and incorporate it with the feed. In future trials, it would be important to see if allicin incorporated in feed has similar anti-parasitic effects. This could also mitigate some potential water pH problems due to treatments. Third, there is need to investigate whether garlic juice used in short-term bath exposures is effective in controlling ichthyophthiriasis and prevents further development of the disease. The final point for future consideration would be to evaluate tissue residues of garlic in the fish flesh and test if palatability is affected.

In conclusion, the present study has demonstrated that garlic and lemon juice have great potential as alternative "safe" treatments for ichthyophthiriasis on tilapia. Both products at concentrations used in the infection trial 1 killed ich theronts and effectively broke the life cycle such that further development of the parasite was prevented. Garlic juice (3g/L) in a continuous static bath (entire trial period) was the most effective treatment while lemon was an effective treatment, but resulted in some water quality problems (reduced pH). The half concentration of garlic juice used in the infection trial 2 was ineffective in treating the infections as it didn't completely remove the infection in the system. Its incorporation in the experimental protocol was in the interest of cost-saving, to see if a much lower concentration could effectively control the infection. In both infection trials, only two variables (condition factor and lymphocyte density) were significantly different among experimental groups, but they exhibited similar results: the infected + untreated (control) fish had lower values. This difference was likely associated with stress among untreated fish from the ich infections. The remaining variables (specific growth rate, feed conversion ratio, neutrophil density, hepatosomatic index and splenosomatic index), although not statistically significant, exhibited consistent patterns: sub –optimal values were obtained among the infected + untreated (control) fish. Overall, those fish infected + garlic treated displayed better growth performance. Interestingly, in the infection trial 2, the uninfected + untreated fish performed similar to infected + garlic treated group, further indicating the efficacy of this bio-product treatment. Therefore the results from the present study indicate that garlic juice (3g/L) in a continuous static bath exposure could be used to effectively mitigate ich infections on tilapia.

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# Appendix A, Tables

Table A1. Summary of some important tilapia species used in fish culture.

| Species | Areas cultured | Salinity tolerance | Growth |
|---|---|---|---|
| *Oreochromis aureus* | Israel,Uganda, Senegal and USA. | Up to 44 ‰ | Maximum attained size is 31.5 cm. |
| *O. mossambicus* | East and Southern Africa, South East Asia, Japan, Latin America, USA and Russia. | Up to 40 ‰ | Males grow faster than females. Maximum size attained is 1.7kg (36 cm) in the wild. |
| *O. niloticus* | Africa, Thailand, Israel and Middle East. | Up to 35 ‰ | Males grow 2 – 5 times faster than females. Maximum size attained in the wild is 2.5kg (50 cm). |
| *Sarotherodon galilaeus* | Israel and North Central Africa. | Up to 29 ‰ | Maximum attained size is 0.8 kg (40 cm). |
| *Tilapia rendalli* | Central West Africa. | Up to 19 ‰ | Reaches 5 – 6cm in 6 weeks. Maximum size attained in the wild is 1.3kg (40 cm). |
| *T. sparmanii* | East and South Africa and Japan | Up to 18 ‰ | Seldom exceeds 100g. Maximum size attained is 300 g (27 cm). |
| *T. zillii* | Widely distributed throughout Africa, Jordan, Syria, and Malaysia | Up to 45 ‰. However doesn't reproduce at salinities of 39 – 44 ‰ | Males grow 35 % faster than females. Maximum size attained is 3 kg (35 cm). |

(Source: Balarin, 1979).

Table A2. Weekly mean water quality parameters measured in Trial 1.

| Dates | pH | DO % saturation | DO (mg/L) | $CO_2$ (mg/L) |
|---|---|---|---|---|
| 11th - 18th Dec, 2003 | 7.0 | 98 | 8.4 | 6 |
| 19th – 26th Dec, 2003 | 7.0 | 93 | 7.5 | 6 |
| 27th Dec – 3rd Jan, 2004 | 7.1 | 90 | 7.4 | 7 |
| 4th – 11th Jan, 2004 | 7.1 | 87 | 7.1 | 8.8 |
| 12th – 19th Jan, 2004 | 7.2 | 79 | 6.8 | 13.8 |
| 20th – 27th Jan, 2004 | 7.2 | 79 | 6.7 | 15.0 |
| 28th Jan – 6th Feb, 2004 | 7.2 | 83 | 7.0 | 12.4 |

| Dates | TAN (mg/L) | $NH_3$ (mg/L) | Temp. (°C) | Nitrite (mg/L) |
|---|---|---|---|---|
| 11th - 18th Dec, 2003 | 0.5 | 0.003 | 25 | 0 |
| 19th – 26th Dec, 2003 | 1.2 | 0.008 | 25.6 | 0 |
| 27th Dec – 3rd Jan, 2004 | 2.1 | 0.02 | 25.3 | 0.05 |
| 4th – 11th Jan, 2004 | 3.0 | 0.02 | 25.4 | 0.05 |
| 12th – 19th Jan, 2004 | 2.8 | 0.03 | 25.6 | 0.28 |
| 20th – 27th Jan, 2004 | 3.0 | 0.03 | 25.8 | 0.30 |
| 28th Jan – 6th Feb, 2004 | 2.8 | 0.03 | 25.6 | 0.15 |

Table A3. Weekly mean water quality parameters measured in Trial 2.

| Dates | pH | DO % saturation | DO (mg/L) | $CO_2$ (mg/L) |
|---|---|---|---|---|
| 28th June- 5th July, 2004 | 6.9 | 89 | 7.7 | 8 |
| 6th – 13th July, 2004 | 7.1 | 84 | 7.4 | 8 |
| 14th – 21st July, 2004 | 7.0 | 86 | 7.5 | 11 |
| 22nd – 29th July, 2004 | 7.1 | 83 | 7.4 | 11 |
| 30th July – 6th Aug, 2004 | 7.1 | 83 | 7.3 | 12 |
| 7th – 14th Aug, 2004 | 7.1 | 81 | 7.1 | 16 |
| 15th – 22nd Aug, 2004 | 7.1 | 82 | 7.3 | 14 |

| Dates | TAN (mg/L) | $NH_3$ (mg/L) | Temp. (°C) | Nitrite (mg/L) |
|---|---|---|---|---|
| 28th June- 5th July, 2004 | 0.8 | 0.01 | 25 | 0 |
| 6th – 13th July, 2004 | 1.7 | 0.02 | 26.1 | 0 |
| 14th – 21st July, 2004 | 2.3 | 0.01 | 25.4 | 0.04 |
| 22nd – 29th July, 2004 | 2.9 | 0.03 | 25.7 | 0.06 |
| 30th July – 6th Aug, 2004 | 3.4 | 0.04 | 25.2 | 0.12 |
| 7th – 14th Aug, 2004 | 3.6 | 0.04 | 25.3 | 0.3 |
| 15th – 22nd Aug, 2004 | 3.6 | 0.04 | 25.6 | 0.3 |

# Appendix B, Figures

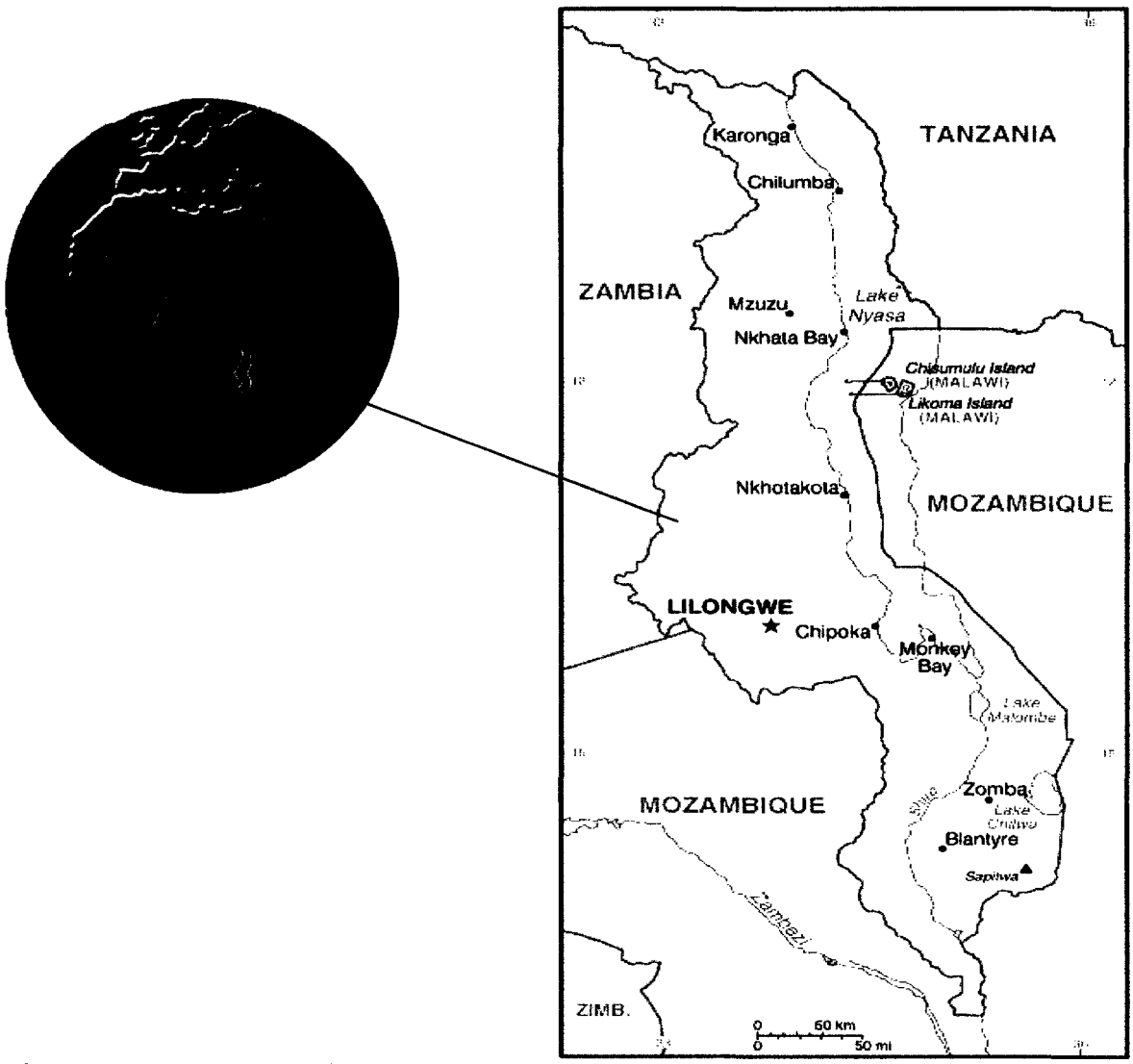


Figure B1. Map of Malawi. (GeographyIQ, 2004)

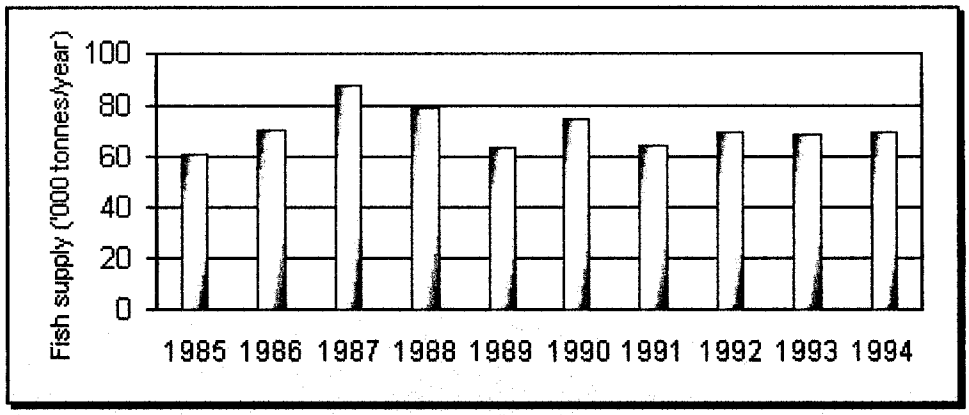


Figure B2. National fish supply ('000 tonnes/year) in Malawi from 1985 - 1994 (State of Environment Report For Malawi, 1998).

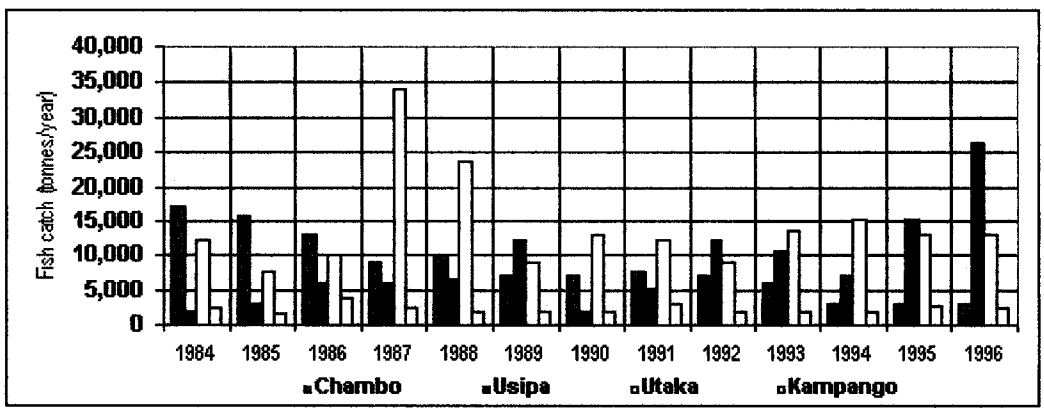


Figure B3. Fish catches (tonnes/year) in Malawi from 1984 - 1996 (State of Environment Report For Malawi, 1998).

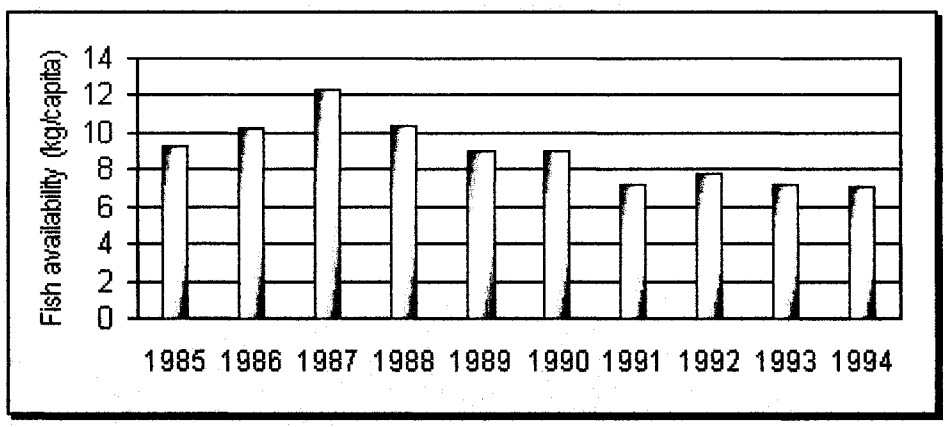


Figure. B4. Fish availability (kg) per capita in Malawi from 1985 - 1994. (State of Environment Report For Malawi, 1998).

Figure B5. Smallholder fish pond culture in Malawi (Stirling Aquaculture, Smallholder Fish Farming in Malawi :)

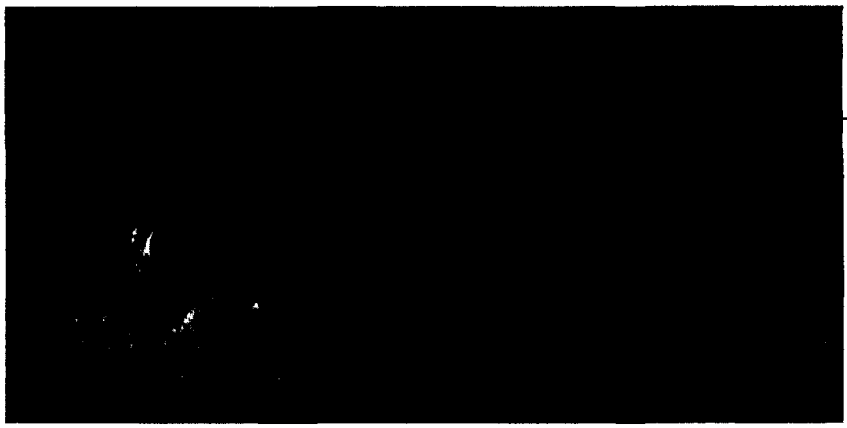


Figure B6. White specks, clinical signs of ichthyophthiriasis (Anonymous, 2004).

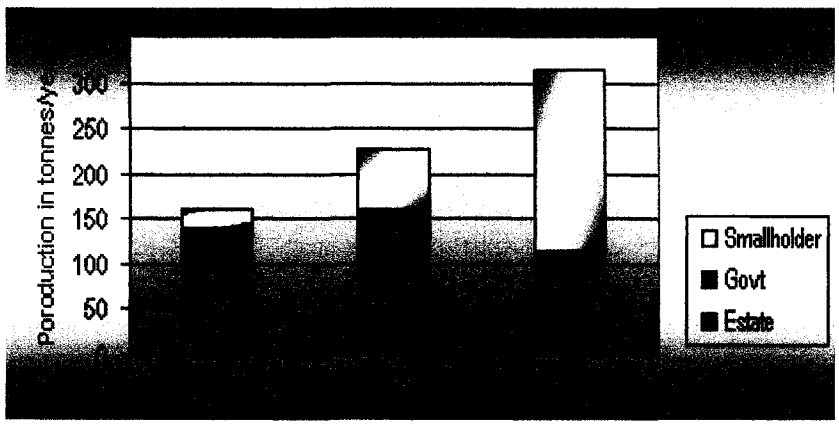


Figure B7. Total aquaculture production in Malawi. (State of Environment Report For Malawi, 1998).

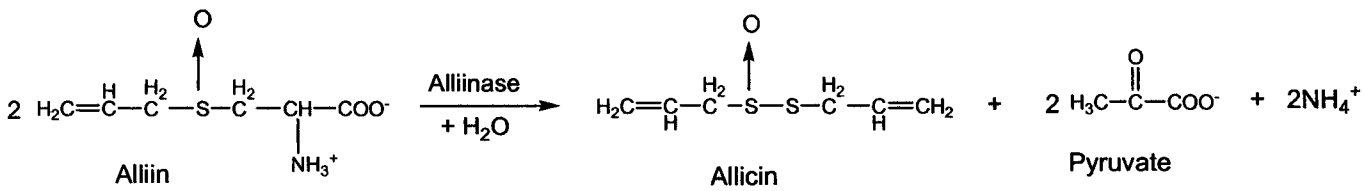


Figure B8. Conversion of alliin to allicin (Miron *et al.* 2002).

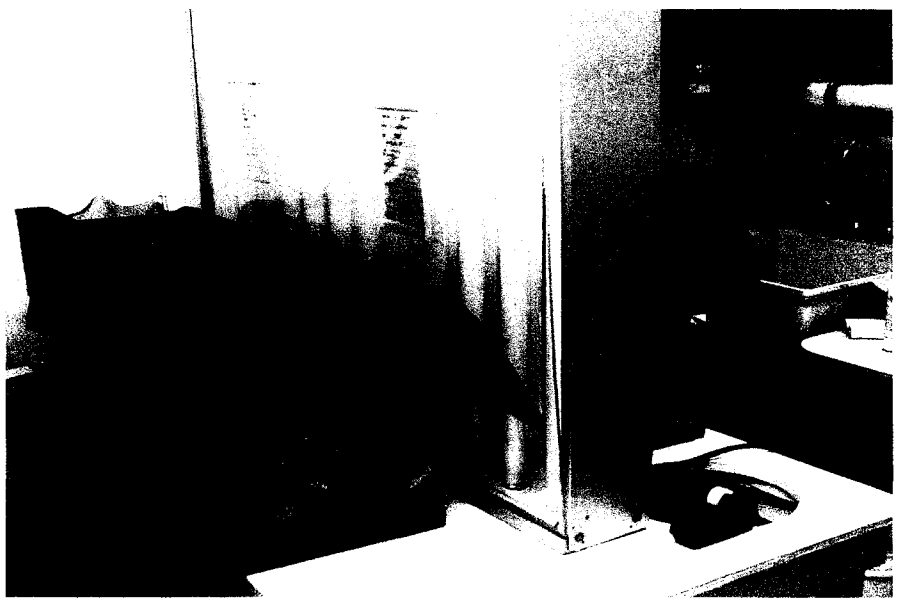

Figure B9. Experimental aquaria (45 cm x 31 cm x 39 cm).