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The evaluation of *Bacillus thuringiensis* subspecies *israelensis* based biopesticides for the control of southern African malaria vectors

Irene Ketseoglou

The evaluation of *Bacillus thuringiensis* subspecies *israelensis* based biopesticides for the control of southern African malaria vectors

Irene Ketseoglou

A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, 2012

Declaration

I declare that this is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any other degree or examination in any other University.

I. Ketseoglou

Irene Ketseoglou

14th day of May 2012

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Abstract

Malaria, one of the most deadly vector-borne diseases in the world, is transmitted by the bite of an infected female *Anopheles* mosquito. Since there are several problems arising from current control programs, alternative control strategies for malaria-carrying vectors are required. The objective of this thesis was to evaluate a recombinant *Anabaena* sp. strain PCC 7120, *Anabaena* PCC 7120#11 (PCC 7120#11), as an agent to control southern African malaria vectors. Select *Bacillus thuringiensis* subspecies *israelensis* (*Bti*) toxin genes, *cry4Aa* and *cry11Aa*, had previously been cloned into *Anabaena* sp. strain PCC 7120.

Evaluation of the potential of PCC 7120#11 and *Bti* as control agents of southern African malaria vectors required the evaluation of the larvicidal activity of PCC 7120#11 and *Bti* against several important malaria vectors. This study showed that all five of the African anopheline species evaluated in laboratory bioassays (*Anopheles gambiae*, *Anopheles arabiensis*, *Anopheles funestus*, *Anopheles quadriannulatus*, and *Anopheles merus*) were susceptible to *Bti*, whereas PCC 7120#11 exhibited high larvicidal activity against four of the five anopheline species. The low larvicidal activity of PCC 7120#11 against *An. funestus* could be due to the absence of the other *Bti* toxins in PCC 7120#11 or differences in the structure and/or density of specific midgut toxin receptors for the Cry4Aa or Cry11Aa proteins.

The Standardized Aquatic Microcosm (SAM), a synthetic multi-species system consisting of a chemically defined medium with the same species and concentration of photosynthetic microorganisms (PMOs) and invertebrates, was used to comprehensively evaluate the effects of *Bti* and PCC 7120#11 in an aquatic environment. The SAM experiments showed that *Bti* was non-toxic to the invertebrates evaluated and did not significantly affect any of the other variables evaluated in the microcosms. *Bti* was shown to be an environmentally friendly control agent.

In the SAM experiments, PCC 7120#11 had a temporary, less than 5 days, statistically significant effect on some of the invertebrate populations and the concentration of phosphate and nitrate in the microcosms. Importantly however, by the end of the experiment there were no significant differences between the control and PCC 7120#11 microcosms for the any of the variables evaluated. PCC 7120#11 did not have any long-term effects on any of the evaluated variables and is thus unlikely to have any long-term effects on water quality or non-target organisms in the field. The larvicidal activity of PCC 7120#11, in the microcosms, decreased considerably within a week, as did the larvicidal activity of *Bti*.

Response surface methodology (RSM), a cost-effective and time-saving technique, was used to evaluate the relationship between the key factors of growth in order to achieve optimal growth of PCC 7120#11. RSM showed that there were complex interactions between the key growth factors (illuminance, air flow, and carbon dioxide), in terms of their effect on optimal growth of PCC 7120#11. The interaction of carbon dioxide and air flow and the interaction of air flow and illuminance had a significant effect on the optimal growth of PCC 7120#11. An optimal volumetric output rate of 0.48 g/l/d was obtained in this study. Different factor level combinations of the key growth factors did not significantly affect the larvicidal activity of PCC 7120#11 against *An. arabiensis* larvae.

This study confirmed the potential of *Bti* as a control agent of all the important anopheline vectors in southern Africa. Furthermore, this study showed that PCC 7120#11 is an environmentally safe larvicidal vector control agent that can be mass produced, in an indoor, flat-plate inclined photobioreactor, and has the potential to be used in integrated vector control programs.

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Chapter 1

General introduction

1.1 Malaria

Malaria is one of the most important parasitic diseases in the world occurring mainly in sub-Saharan Africa, large areas of Latin America, India, South East Asia, and Western Pacific (Kager, 2002; Ashley *et al*., 2006). Malaria is a vector-borne disease caused by a protozoan parasite of the genus *Plasmodium* (Sherman, 2001; Ashley *et al*., 2006; Cowman & Crabb, 2006). *Plasmodium* species infect a broad range of hosts but only five species are known to infect humans: *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium falciparum*, *Plasmodium knowlesi*, and *Plasmodium ovale* (Phillips, 2001; Ashley *et al*., 2006; Vythilingam, 2010). *P. vivax* is widespread in Central and South America and Asia (Breman, 2001; Sherman, 2001). *P. ovale* and *P. falciparum* are found in Africa, with *P. falciparum* predominately occurring in sub-Saharan Africa (Breman, 2001).

There are an estimated 300 to 500 million clinical cases of malaria worldwide (Sachs & Malaney, 2002) and over 1.2 million malaria-related deaths reported each year, with most deaths being caused by *P. falciparum* (Murray *et al*., 2012). The symptoms of malaria include fever, headaches, tiredness, abdominal pains, diarrhoea, vomiting, and low blood sugar (WHO, 2000; Sherman, 2001; Ashley *et al*., 2006). In the more severe cases, the symptoms include kidney failure, fluid on the lungs, delirium, and coma (WHO, 2000). More than 90% of total malaria related deaths occur in sub-Saharan Africa, with pregnant women and children below the age of five being the most susceptible (Schwartlander, 1997; Sherman, 2001; Touré *et al*., 2004).

Unfortunately, the greatest impact of malaria tends to occur in developing countries, such as those in sub-Saharan Africa (Sachs & Malaney, 2002). Malaria affects a society in many ways including reducing population growth as a result of early mortality and birth defects, increasing school absenteeism, and increasing medical costs to the state and individual. Thus the direct and indirect costs of malaria in Africa are estimated to exceed two billion U.S

dollars per year (Kager, 2002; Sachs & Malaney, 2002). As an added concern, co-infection with malaria can adversely influence the severity of other pathogens such as human immunodeficiency virus (HIV) (Kager, 2002). Thus, it is of utmost importance to control the disease or more importantly, the spread of the disease.

1.2 Life cycle of *Plasmodium* species

The different *Plasmodium* species vary slightly in their life cycles with *P. falciparum* usually taking 6 to 14 days to show symptoms (Phillips, 2001). The life cycle of *Plasmodium* species consists of a sexual and an asexual phase (Talman *et al*., 2004). In brief, sporozoites from the salivary glands of an infected female *Anopheles* mosquito are injected into the blood of the host and subsequently invade the liver cells (Sidjanski & Vanderberg, 1997; Cowman & Crabb, 2006). The sporozoites, after forming a parasitophorous vacuole, undergo asexual multiplication for approximately 6 to 15 days resulting in the production of thousands of merozoites (Phillips, 2001; Cowman & Crabb, 2006). The merozoites burst from the liver cells, invade the erythrocytes and then undergo asexual reproduction to produce a blood-stage schizont (Cowman & Crabb, 2006). The schizont releases its merozoites, destroying the erythrocytes and then proceeds to invade more erythrocytes (Ashley *et al*., 2006). This asexual cycle usually lasts for 48 or 72 hours, depending on the *Plasmodium* species (Phillips, 2001).

Not all the merozoites develop asexually; some of the merozoites differentiate into sexual forms, micro- and macrogametocytes (Talman *et al*., 2004). These gametocytes will be ingested by another female *Anopheles* mosquito, when feeding on an infected individual (Ashley *et al*., 2006). Within the mosquito midgut, the gametocytes undergo gametogenesis, with the motile microgametes fertilizing the macrogamete (Ashley *et al*., 2006). The resulting zygote burrows through the midgut wall and encysts on the exterior of the gut wall as an oocyst (Phillips, 2001). Within the developing oocyst, sporozoites develop and are released into the mosquito body cavity when the oocyst

ruptures (Phillips, 2001). The sporozoites then migrate to the salivary gland to complete the cycle, approximately 7 to 18 days after gametocyte ingestion (Phillips, 2001).

1.3 Malaria vectors

There are roughly 400 species of *Anopheles*, 60 of which transmit malaria but only 30 are of major importance (Bruce-Chwatt, 1985). The major vectors of interest in this study belong to the *Anopheles gambiae* Giles complex and the *Anopheles funestus* Giles group (Gillies & De Meillon, 1968). There are other important malaria vectors such as *Anopheles nili* and *Anopheles moucheti* (Fontenille & Simard, 2004; Antonio-Nkondjio *et al*., 2009), but these vectors were not evaluated in this thesis.

1.3.1 The *An. gambiae* Giles complex

The mosquitoes in the *An. gambiae* Giles complex are the most efficient vectors of malaria worldwide (Coetzee, 2004). It comprises seven named species, one unnamed species, and several "incipient" species (Coetzee *et al*., 2000; Fontenille & Simard, 2004; Moreno *et al*., 2007). The seven named species are *Anopheles gambiae* Giles *sensu stricto*, *Anopheles arabiensis* Patton, *Anopheles quadriannulatus* Theobald (species A and B), *Anopheles bwambae* White, *Anopheles merus* Dönitz, and *Anopheles melas* Theobald (Gillies & Coetzee, 1987; Hunt *et al*., 1998; Coetzee *et al*., 2000).

Members of the *An. gambiae* complex have been reported from most countries of Africa and its adjacent islands, including Madagascar (Coetzee *et al*., 2000). *An. melas*, an important malaria vector in the West African coast, prefers to breed in salty, swampy waters (Fontenille & Simard, 2004). *An. merus*, found on the East African coast, breed in brackish lagoons, ponds, swamps, pools and puddles that are flooded at spring-tide (White, 1974). The *An. merus* and *An. melas* adults exhibit exophilic (rest outdoors in a variety of shelters) behaviour and are mainly zoophilic (feeds on animals) (White, 1974).

An. bwambae, mainly in Uganda, breeds in mineral hot springs whereas *An. arabiensis, An. quadriannulatus*, and *An. gambiae* are found in freshwater (Gillies & Coetzee, 1987). *An. bwambae* and *An. quadriannulatus* have limited roles as malaria vectors, mainly due to their restricted geographic distribution (*An. bwambae)* and their zoophilic nature (*An. quadriannulatus)* (Fontenille & Simard, 2004). Often efficient malaria vectors and non vector species are found within the same complex (Fontenille & Simard, 2004).

An. arabiensis and *An. gambiae s.s.*, arguably the two most efficient malaria vectors, are widespread throughout sub-Saharan Africa and surrounding islands (Hargreaves *et al*., 2003; Coetzee, 2004; Fontenille & Simard, 2004). They generally coexist in their distribution but *An. gambiae s.s.* is usually found in more humid environments (major vector in tropical Africa) than *An. arabiensis* (major vector in southern Africa) (Coetzee *et al*., 2000). Their behaviours also vary slightly with *An. gambiae s.s.* being highly anthropophilic (feeds on humans) and endophilic (rests indoors) (Gillies & De Meillon, 1968), whereas the females of *An. arabiensis* exhibit variable behaviour, being both anthropophilic and zoophilic, as well as endophilic and exophilic (Gillies & Coetzee, 1987). The variability in the feeding and resting behaviour of *An. arabiensis* reduces the success rate of several control programs (Collins & Besansky, 1994). Several vectors often transmit malaria together, further complicating control programs (Fontenille & Simard, 2004).

The *An. gambiae s.s.* larvae are often found in shallow, sun-lit pools (such as hoof-prints, car-tracks, and drains), vegetated ponds, irrigation channels, permanent wells, temporary pools, and the edges of swamps (Gillies & De Meillon, 1968). *An. arabiensis* larvae tend to breed in small, sunny, clear, shallow, temporary puddles or pools (Gillies & Coetzee, 1987).

1.3.2 The *An. funestus* Giles group

The *An. funestus* Giles group comprises nine species, these being *Anopheles funestus* Giles, *Anopheles parensis* Gillies, *Anopheles vaneedeni* Gillies and Coetzee, *Anopheles leesoni* Evans, *Anopheles fuscivenosus* Leeson, *Anopheles aruni* Sobti, *Anopheles rivulorum* Leeson, *Anopheles brucei* Service, and *Anopheles confusus* Evans and Leeson (Gillies & De Meillon, 1968). These nine species are very morphologically similar in their adult stage (Gillies & De Meillon, 1968). *An. funestus* is the most important vector of malaria parasites in this group, being highly anthropophilic and endophilic (Coetzee & Fontenille, 2004). *An. funestus* feeds in the night but has also been known to bite in the early hours of the morning and during the day in dark houses (Gillies & De Meillon, 1968). The larvae tend to thrive in large, permanent water bodies such as weedy sides of streams, rivers, and furrows (Gillies & De Meillon, 1968). *An. funestus* has a wide distribution throughout Africa, extending from northern Sudan to South Africa and across West Africa to northern Mali and Senegal (Gillies & De Meillon, 1968; Fontenille & Simard, 2004). It is responsible for causing one of the largest malaria epidemics in South Africa (Hargreaves *et al*., 2000; Coetzee, 2006).

The other species of the group are more localized in their distribution, with *An. parensis* and *An. confusus* being found in East Africa (Kenya and Tanzania) and KwaZulu Natal in South Africa (Gillies & De Meillon, 1968). *An. vaneedeni* has been recorded in the northern areas of South Africa, *An. aruni* in Zanzibar, *An. fuscivenosus* in Zimbabwe, and *An. brucei* in Nigeria (Gillies & De Meillon, 1968; Gillies & Coetzee, 1987). *An. rivulorum* and *An. leesoni* occur in Ethiopia, West Africa, and northern parts of South Africa (Gillies & De Meillon, 1968). These species are mainly zoophilic, with only *An. rivulorum* considered to be a minor vector in Tanzania (Wilkes *et al*., 1996).

1.3.3 The *An. nili* group and *An. moucheti*

Other secondary but important vectors of malaria are the *An. nili* group and *An. moucheti* Evans (Fontenille & Simard, 2004; Antonio-Nkondjio *et al*., 2009). The *An. nili* group consists of four recognized species: *An. nili* Theobold, *An. somalicus* Rivola and Holstein, *An. ovengensis* Awono-Ambene, Kengne, Simard, Antonio-Nkondjio, and Fontenille, and *An. carnevalei* Brunhes, Le Goff, and Geoffroy (Awono-Ambene *et al*., 2006). Members of this group, in particular *An. nili* are efficient malaria vectors in the equatorial forests of central Africa (Antonio-Nkondjio *et al*., 2009). *An. nili* prefer to breed in vegetation or dense shade along fast moving streams and along edges of streams and rivers (Gillies & De Meillon, 1968). *An. nili* are known to exhibit anthropophilic and exophilic behaviour (Antonio-Nkondjio *et al*., 2009). *An. ovengensis* and *An. carnevalei* are also forest mosquitoes, however few studies have evaluated this group of malaria vectors in detail (Antonio-Nkondjio *et al*., 2009).

An. moucheti, found in the forests of central Africa, breed in slow moving streams and large rivers of central Africa (Gillies & De Meillon, 1968). *An. moucheti* is anthropophilic and tends to be endophilic (Antonio-Nkondjio *et al*., 2009). Although it is an efficient vector of malaria, very few studies have been performed on *An. moucheti* (Fontenille & Simard, 2004).

1.4 Developmental stages of the *Anopheles* mosquito

The developmental stages of the anophelines include the egg, larval, pupal, and adult stages (Service, 2008).

1.4.1 Egg stage

The anopheline eggs, approximately 50 to 200 per female per batch, are laid singly on the surface of the water (Priest, 1992). The eggs are small, black boat-shaped, and on either side of the egg they have air-filled sacs called floats (Service, 2008). The eggs are not able to withstand desiccation but

provided the right conditions are prevalent, they hatch within 2-3 days (Priest, 1992).

1.4.2 Larval stage

The larvae are surface feeders and are known to feed on algae, bacteria, yeasts, protozoans, and other microorganisms (Merritt *et al*., 1992; Priest, 1992). The anopheline larvae lack a respiratory siphon and thus use a pair of well-developed palmate hairs on their segmented abdomen to help them lie parallel to the surface of the water (Service, 2008). There are four active larval instars or stages (Priest, 1992). At the end of each instar, the larvae molt and after the fourth instar, the larvae metamorphose into pupae (Priest, 1992; Service, 2008).

1.4.3 Pupal stage

All anopheline pupae are aquatic and comma-shaped (Service, 2008). Pupae do not feed but breathe near the water surface (Service, 2008). Pupal development depends on the climate with pupae developing faster in warmer climates (Service, 2008). At the end of the pupal life, an adult mosquito emerges (Service, 2008).

1.4.4 Adult stage

The adult mosquitoes are slender, and small, with their body divided into three sections; the head, the thorax, and the abdomen (Service, 2008). The head has a forward-projecting proboscis, which contains the piercing mouth parts (Service, 2008). The thorax is covered with scales, which are arranged in distinctive patterns for the different species (Service, 2008). The abdomen becomes distended after the females have a blood-meal (Service, 2008). The blood serves as a source of protein for the production of eggs (Service, 2008).

Several control programs target either the adult and/or the larval stages of the anopheline mosquitoes (Priest, 1992; Hougard *et al*., 2002; Walker & Lynch, 2007).

1.5 Malaria vector control strategies

Effective control strategies for malaria vectors pose a great challenge as no single strategy is applicable for all situations (Rose, 2001; Takken & Knoll, 2008). The most important factors that have to be taken into account in vector control strategies include the species of the mosquito vector, their behaviour, their developmental stage, and their susceptibility to insecticides (Phillips, 2001; Fontenille & Simard, 2004; Walker & Lynch, 2007).

Malaria control is multi-faceted, with primary control strategies being environmental management or modification, which includes reducing or eliminating mosquito breeding sites; destroying larval or adult stages; and reducing human-mosquito contact (Davidson, 1982; WHO, 1995; Rose, 2001; Kager, 2002). Unfortunately, eliminating or reducing mosquito breeding sites is almost an impossible task as new breeding sites constantly emerge, especially in areas that experience ample rainfall (Phillips, 2001; Rose, 2001).

Secondary control strategies involve early diagnosis and prompt treatment of malaria in people in high risk areas (WHO, 1995; Kager, 2002; Giao *et al*., 2005). Anti-malarial drugs such as the quinolines and arylaminoalcohols, the antifols, the artemisnin derivatives, antibacterial agents, and the hydroxynaphthaquinones have been used to control the incidence of malaria for almost 50 years (Ashley *et al*., 2006). In developing countries, however, early diagnosis is not always possible (Shiff, 2002). Furthermore, resistance to the current anti-malarial drugs has been reported, with new alternative drugs often being unaffordable in developing countries (Shiff, 2002).

The third control strategy is early detection of epidemics and rapid application of control measures (Phillips, 2001). The strategies involving destroying the larval or adult stages, and reducing human-mosquito contact, require the use of chemical and biological control agents or biopesticides (Phillips, 2001).

Integrated vector control programs are currently being implemented for the control of mosquitoes in several parts of the world. Integrated vector control programs involve the integration of chemical and biological vector control programs, with the removal of larval breeding sites and other disease control measures (Fillinger *et al*., 2004; Beier *et al*., 2008). Integrated vector control programs also incorporate traditional control methods, such as chemical and biological vector control programs, with decision-making based on human and institutional resources, surveillance, and sustainability in the communities (Rose, 2001; Beier *et al*., 2008).

1.5.1 Chemical insecticides

Successful eradication of malaria in parts of the world was achieved mainly by targeting the larval or adult stages of the mosquitoes (Walker & Lynch, 2007). Chemical insecticides can be used against the adult and larval stages of the mosquitoes (Coosemans & Carnevale, 1995). Chemical insecticides may be broadly classified into five groups: petroleum oils and derivatives; the pyrethroids; the organophosphates such as malathion and temephos; the organochlorines, which include dichlorodiphenyltrichloroethane (DDT); and the carbamates (Phillips, 2001).

Adult mosquitoes are usually controlled by indoor residual spraying, insecticide- treated fabrics like bed nets and curtains, and space sprays (Coosemans & Carnevale, 1995; Ashley *et al*., 2006; N'Guessan *et al*., 2007; Walker & Lynch, 2007). Indoor residual spraying (IRS) involves spraying the inside of human dwellings with organochlorines, organophosphates, pyrethroids or carbamates (N'Guessan *et al*., 2007; Mabaso *et al*., 2004). IRS

is based on the principal that the female *Anopheles* mosquito digests the blood meals in certain resting places (Coosemans & Carnevale, 1995). If the resting places are indoors (mosquitoes exhibiting endophilic behaviour), the *Anopheles* mosquito is likely to come into contact with the treated surfaces. IRS is only effective against vectors that exhibit endophillic feeding behaviours (Gillies & Coetzee, 1987). Although IRS was shown to control outbreaks in sub-Saharan Africa and Equatorial Guinea (Sharp *et al*., 2007), a lack of organization, sustainability, and resistance to the spraying from dwellers reduced their efficacy (Goodman *et al*., 2001; Hougard *et al*., 2002).

Insecticide-treated fabrics in particular bed nets (ITN) are designed to reduce the contact of the vector with humans (Coosemans & Carnevale, 1995; Kazura, 2003; N'Guessan *et al*., 2007). ITN kills the vector before they bite the host, thereby providing protection against the vector (Coosemans & Carnevale, 1995). Photo-stable pyrethroids, such as permethrin and deltamethrin, are used because of their persistence and relative safety to humans (Githeko *et al*., 1996; Hougard *et al*., 2002; Shiff, 2002; Okoye *et al*., 2008). ITN have also been shown to be effective but large-scale implementation, adult mosquito avoidance, affordability, retreating of the nets, and sustainability issues such as limited human and financial resources pose a serious problem for their continued use (Goodman *et al*., 2001; Shiff, 2002; Hougard *et al*., 2002; Killeen *et al*., 2002; Touré *et al*., 2004).

Space spraying involves thermal fogging or cold aerosol sprays that result in small droplets of insecticide being suspended in the air (Davidson, 1982; Rose, 2001). This leads to the death of mosquitoes as they come into contact with the small droplets (Coosemans & Carnevale, 1995; Rose, 2001). Space spraying is generally applied during epidemic outbreaks and requires repeated application (Davidson, 1982; Coosemans & Carnevale, 1995).

Although targeting adult female mosquitoes with the above methods reduces the spread of malaria, there are several disadvantages. These include the complexities of maintaining IRS programs, sufficient coverage of houses in the communities, limited financial resources to spray dwellings and supply ITNs, compliance of using ITNs, and avoidance behaviours of the vectors towards the insecticides (Davidson, 1982; Kager, 2002; Killeen *et al*., 2002). As a consequence, some control programs (not in southern Africa) focus on mosquito larval control strategies (Killeen *et al*., 2002).

The development and implementation of effective larval control strategies requires adequate information about the distribution of water bodies, breeding sites of the larvae, and behaviour of vector larvae (Rozendaal, 1997; Walker & Lynch, 2007). The breeding sites or water bodies should preferably be relatively small, easily accessible, and scarce (Rozendaal, 1997; Walker & Lynch, 2007).

Petroleum oils were applied to water bodies over 100 years ago to asphyxiate larvae (Gratz & Pal, 1988). Paris green (copper acetoarsenite) was also used extensively on water bodies (Davidson, 1982; Sherman, 2001), but due to the risks posed by its high toxicity, it is no longer recommended (Coosemans & Carnevale, 1995). DDT was used, in the 1940s and 1950s, to control malaria vectors in the U.S.A., Venezuela, Italy, India, and in southern Africa (Sherman, 2001; Hargreaves *et al*., 2003). Due to its long-term persistence and detrimental effects on non-target organisms, alternatives to DDT are recommended (Walker & Lynch, 2007). Several organophosphate-based larvicidal formulations and synthetic pyrethroids are also effective larvicides but high toxicity to aquatic non-target organisms renders them unfavourable control agents (Gratz & Pal, 1988).

The main disadvantage of using chemical insecticides in the control of malaria vectors, both adult and larval stages, is the development of resistance

(Ranson *et al*., 2001; Rose, 2001; Hougard *et al*., 2002; Shiff, 2002; Coetzee, 2004; N'Guessan *et al*., 2007). Important malaria vector species such as *An. gambiae*, *An. funestus*, and *An. arabiensis* have exhibited resistance to insecticides such as DDT, dieldrin, carbamates, and pyrethroids in various epidemiological and environmental settings (Hargreaves *et al*., 2003; N'Guessan *et al*., 2007; Abdalla *et al*., 2008; Okoye *et al*., 2008; Awolola *et al*., 2009; Mouatcho *et al*., 2009; Vezenegho *et al*., 2009). Resistance to insecticides is predominantly mediated by the detoxification of insecticides by enzymes before they reach their target sites; by a change in the sensitivity of the target site that reduces the binding of the insecticide; and reduction of penetration of the insecticide to the active site (Davidson, 1982; Hemingway & Ranson, 2000; Ranson *et al*., 2000; Hemingway *et al*., 2004; Liu *et al*., 2006). Another major disadvantage of using chemical insecticides is the detrimental effects on the environment and non-target organisms (Gratz & Pal, 1988; Rose, 2001; Federici *et al*., 2003).

1.5.2 Biological control

The use of biological control agents or biopesticides offers an environmentally friendly strategy in the control of malaria vectors (Takken & Knols, 2008). Other advantages to using biological control agents include their effectiveness at low doses, safety to humans and non-target organisms, increased target specificity, and low risk of the development of resistance (Yap, 1985; Stevens *et al*., 1994; Lacey *et al*., 2001; Fourcy *et al*., 2002; Killeen *et al*., 2002; Federici *et al*., 2003).

Biological control agents used to control malaria vectors include fungi, nematodes, larvivorous fish, and bacteria (Petersen & Willis, 1974; Davidson, 1982; Homski *et al*., 1994; Cuda *et al*., 1997; Lacey *et al*., 2001; Pérez-Pacheco *et al*., 2005; Scholte *et al*., 2005; Crickmore, 2006). Mermithid nematodes, *Romanomermis culicivorax* and *Diximermis peterseni*, were shown to parasitize and kill mosquito larvae (Platzer, 1981; Giblin & Platzer,

1985). The shortcomings of using nematodes is that invertebrates prey on the nematodes thus requiring high and continual inoculations of nematodes (Petersen & Willis, 1974; Pérez-Pacheco *et al*., 2005). Larvivorous fish, such as *Gambusia affinis* and *Aphanius dispar*, have also been used as agents to control malaria vectors but the fish would be preyed upon by the predators in the environment (Davidson, 1982; Homski *et al*., 1994). Control would require reintroduction of large numbers of the larvivorous fish, thereby becoming a costly time-consuming control method (Davidson, 1982; Homski *et al*., 1994). Although larvivorous fish have been used successfully as biological control agents (Martínez-Ibarra, 2002), larvivorous fish may devastate indigenous freshwater organisms, producing undesirable changes in the ecosystem (Davidson, 1982; Homski *et al*., 1994; Rose, 2001). Entomopathogenic fungi, such as *Metarhizium anisopliae* and *Lagenidium giganteum*, have also been evaluated as control agents of malaria vectors (Cuda *et al*., 1997; Scholte *et al*., 2003; Scholte *et al*., 2005). These fungi were shown to be effective against endophilic adult mosquitoes, however further field testing is required (Scholte *et al*., 2005; Takken & Knols, 2008). Bacteria have been successfully and extensively used for many years worldwide to control mosquitoes (Priest, 1992; Mulla, 1994), with the most commonly used bacterium in biological control of mosquitoes being *Bacillus sphaericus* and *Bacillus thuringiensis* (Lacey *et al*., 2001; Crickmore, 2006).

B. sphaericus produces a single binary toxin (Bin) during sporulation, that is composed of two proteins, the binding (BinB) and toxin (BinA) domains (Federici *et al*., 2003; Opota *et al*., 2008). There is varied susceptibility to *B. sphaericus* amongst mosquito species, with the *Culex* species being the most susceptible (Davidson, 1989; Berry *et al*., 1993; Opota *et al*., 2008). The *Anopheles* species exhibited intermediate susceptibility with *B. sphaericus* (Davidson, 1989), hence this study focussed on *Bacillus thuringiensis* based biopesticides.

1.5.3 *Bacillus thuringiensis (Bt)*

Bacillus thuringiensis Berliner (*Bt*) was isolated from a flour moth in the German province of Thuringia and was described by Berliner in 1915 (Milner, 1994). It was originally found in Japan in 1902 but it was poorly described leading to the Berliner description and name being accepted as the original (Milner, 1994). *Bt* is a spore-forming, Gram-positive, ubiquitous, soil bacterium that produces insecticidal, crystalline protein inclusions (Schnepf *et al*., 1998). The insecticidal, crystalline protein inclusions are known as delta (δ) endotoxins (Schnepf *et al*., 1998; Khasdan *et al*., 2001). In addition to the δ-endotoxins, vegetative insecticidal proteins (VIPs), alpha (α-), and beta (β-) exotoxins are produced (Lüthy, 1980; Levinson *et al*., 1990; Cooper, 1994; Estruch *et al*., 1996). The VIPs do not form parasporal crystal proteins (Schnepf *et al*., 1998). They are expressed in the vegetative stage of growth and then secreted from the cell (Estruch *et al*., 1996; Crickmore, 2006). They are toxic to lepidopteran insect larvae (Estruch *et al*., 1996). The α-exotoxin is a heat sensitive 50 kilodalton (kDa) compound that is produced during the vegetative growth phase of *Bt* (Lüthy, 1980). The β-exotoxin or thuringiensin, is a heat-stable insecticidal adenosine triphosphate (ATP) analog that is produced during the vegetative phase of growth (Vaňková, 1978; Lüthy, 1980; Cooper, 1994). β-exotoxin acts by inhibiting DNA-directed RNA polymerase by competing with ATP for binding sites (Sebesta & Sternbach, 1970). Unlike the δ-endotoxins, β-exotoxin has a broad host range (Lüthy, 1980).

The δ-endotoxins comprise two multi-genic families, *cry* and *cyt* (Soberón *et al*., 2007). A *Bt* strain can produce between one and five crystal toxins, packaged into single or multiple crystals (De Maagd *et al*., 2001). The genes encoding the crystal proteins are found on transmissible plasmids with the production of the toxin crystal being coupled to sporulation (Schnepf *et al*., 1998; Crickmore, 2006). The crystal (Cry) protein toxins have a defined spectrum of insecticidal activity (De Maagd *et al*., 2001). They have been shown to be toxic to the orders Lepidoptera, Diptera, Coleoptera,

Hymenoptera, Homoptera, Orthoptera, Mallophaga, and the nematodes (Goldberg & Margalit, 1977; Margalit & Dean, 1985; Feitelson *et al*., 1992; Milner, 1994; Crickmore *et al*., 1998; Marroquin *et al*., 2000; De Maagd *et al*., 2001). The cytolytic (Cyt) protein toxins are active against the order Diptera (Butko, 2003).

The crystals are solubilized in the presence of the alkaline conditions (pH >9.5) of the insect's midgut to form protoxins (Tojo & Aizawa, 1983). The protoxins are then cleaved by the insect's digestive proteases to form to a protease-resistant core (active Cry toxins) (Höfte & Whiteley, 1989; Crickmore, 2006). The activated Cry toxins have two important functions, receptor binding and ion channel activity (Schnepf *et al*., 1998). The activated Cry toxins are composed of three functional domains; a seven α-helical bundle that is involved in membrane insertion (domain I), three β-sheets having a "Greek-key" conformation (domain II), and two, twisted, anti-parallel β-sheets in a "jelly-roll" formation (domain III) (Schnepf *et al*., 1998; De Maagd *et al*., 2001; De Maagd *et al*., 2003; Soberón *et al*., 2007). Domain I is involved in membrane insertion and pore formation, whereas domains II and III are involved in receptor recognition and binding (Ge *et al*., 1989; Schnepf *et al*., 1990; De Maagd *et al*., 2001; De Maagd *et al*., 2003). Domain III also plays a role in pore formation (De Maagd *et al*., 2001). Binding is a two-stage process involving reversible (the toxin recognizes and binds to the receptors) and irreversible steps whereby the toxin inserts into the cell membrane (Hofmann *et al*., 1988; Rajamohan *et al*., 1995). The activated Cry toxins interact with specific protein receptors on the insect's apical brush border of the midgut epithelium (Hofmann *et al*., 1988; Davidson, 1989; Van Rie *et al*., 1990; Ravoahangimalala *et al*., 1993; Ravoahangimalala & Charles, 1995). Once the toxin has bound to the receptor it inserts into the apical membrane of the columnar epithelial cells, causing ion channels or pores to form (Thomas & Ellar, 1983; Schnepf *et al*., 1998). The Cyt proteins, which are composed of a single αβ domain (Butko, 2003), also aid in pore formation by

interacting with the membrane lipids and then inserting into the membrane (Promdonkoy & Ellar, 2003). The pores lead to the lysis of the cells allowing the alkaline gut contents to escape into the hemolymph, paralyzing the insect's digestive system resulting in insect death (Höfte & Whiteley, 1989; Schnepf *et al*., 1998).

Many *Bt* strains with different insect host spectra have been classified into different subspecies based on their flagellar H-antigen (Höfte & Whiteley, 1989; Kaur, 2000). There are more than 40 subspecies with the more commonly used subspecies being *Bacillus thuringiensis* subspecies *israelensis* (Priest, 1992; Crickmore, 2006).

1.5.4 *Bacillus thuringiensis* subspecies *israelensis* (*Bti*)

Bacillus thuringiensis subspecies *israelensis* (*Bti*) was found in Israel in 1976 and is widely used in biological control (Margalit & Dean, 1985; Karch *et al.*, 1991; Becker & Rettich, 1994; Fillinger *et al*., 2003). *Bti* produces crystalline inclusions that contain highly toxic Cry and Cyt proteins during sporulation (Takken & Knols, 2008). *Bti* produces three major inclusion types (Ibarra & Federici, 1986). The crystals within these inclusions are composed of at least six polypeptides of varying sizes; Cry4Aa (134 kDa), Cry4Ba (128 kDa), Cry10Aa (58 kDa), Cry 11Aa (70 kDa), and Cyt1Aa (27 kDa) and Cyt2Ba (29 kDa) (Höfte & Whiteley, 1989; Ben-Dov *et al*., 1999; Berry *et al*., 2002). The genes encoding for these polypeptides are located on one of the largest (137 kb) transmissible plasmid (pBtoxis) (Faust *et al*., 1983; Ben-Dov *et al*., 1996). The plasmid also encodes for two proteins, P19 and P20 (Manasherob *et al*., 2001). Synergistic interactions exist with all combinations of toxins or polypeptides being used, with toxicity depending on the combinations (Tabashnik, 1992; Wu *et al*., 1994; Poncet *et al*., 1995; Wirth *et al*., 1997; Pérez *et al*., 2005; Otieno-Ayayo *et al*., 2008). However, little is known about the exact mechanism of the synergistic interactions (Schnepf *et al*., 1998).

Studies have examined the larvicidal activity of *Bti* against Afrotropical anophelines in West Kenya (Fillinger *et al*., 2003); in Zaire (Karch *et al.*, 1991); and in Burkina Faso (Skovmand & Sanogo, 1999), just to mention a few. A major advantage of using *Bti* as a biopesticide is that non-target organisms are not capable of activating the toxin because the midgut pH may not be the required pH and/or the specific midgut receptors may not be present (Becker, 1997). There is also low risk of resistance being developed to *Bti* due to the specific conditions during which it is activated, and resistance would have to develop to all the Cry and Cyt proteins that make up the endotoxin (Stevens *et al*., 1994; Wirth *et al*., 1997; Fourcy *et al*., 2002; Takken & Knols, 2008).

There are, however a few disadvantages to using *Bti* as a control agent mainly its low persistence in the field (Boussiba *et al*., 2000; Manasherob *et al*., 2002). The most important issues governing the low persistence and hence low larvicidal activity of *Bti* preparations or formulations are that the spores and crystals of the *Bti* sediment rapidly from the mosquito larval feeding zone posing a problem for *Anopheles* larvae that feed at the water surface (Mulla *et al*., 1982; Merritt *et al*., 1992). The *Bti* spores and crystals tend to adsorb to particles in the water body (Ignoffo *et al*., 1981; Ramoska *et al*., 1982; Ohana *et al*., 1987) and they also get inactivated by UV (Griego & Spence, 1978; Ignoffo & Garcia, 1978; Becker *et al*., 1992). Another issue with using *Bti* in the field is the ingestion by other water organisms and the lack of *Bti* production in aquatic environments (Priest, 1992; Mulla *et al*., 1990; Blaustein & Margalit, 1991; Becker *et al*., 1992).

Researchers have attempted to overcome some of the problems associated with *Bti* by cloning the genes for the *Bti* toxins into aquatic microorganisms such as cyanobacteria and bacteria (Porter *et al*., 1993; Armengol *et al*., 2005; Crickmore, 2006). Aquatic microorganisms are found in the larval feeding/breeding zones of mosquitoes, are buoyant, are a food source for

larvae, and are resistant to UV damage and other harsh environmental conditions (Thiery *et al*., 1991; Murphy & Stephens, 1992; Tandeau de Marsac & Houmard, 1993; Suzuki & Giovannoni, 1996; Manasherob *et al*., 2002; Crickmore, 2006; Rey *et al*., 2009).

1.5.5 Toxin expressing recombinant microorganisms

Genetically engineered microorganisms such as cyanobacteria and other aquatic Gram-negative bacteria have been evaluated as delivery vehicles for mosquitocidal toxins (Federici *et al*., 2003). Select *Bti cry* genes have been transferred into several aquatic microorganisms such as *Caulobacter crescentus*, *Ancylobacter aquaticus*, *Asticcacaulis excentricus*, *Synechococcus* sp., *Enterobacter amnigus*, *Synechocystis,* and *Anabaena* sp. (Yap *et al*., 1994a; Yap *et al*., 1994b; Liu *et al*., 1996; Wu *et al*., 1997; Boussiba *et al*., 2000; Khasdan *et al*., 2003; Tanapongpipat *et al*., 2003; Stevens *et al*., 1994; Armengol *et al*., 2005; Zheng *et al*., 2007). However several of these microorganisms were laboratory strains and some were not found in the midgut of mosquito larvae (Tanapongpipat *et al*., 2003). Furthermore, low larvicidal activity was observed against the mosquito species, with LC_{50} values ranging from 6.83 x 10^5 cells/ml to 4.1 x 10^7 cells/ml (Stevens *et al*., 1994; Yap *et al*., 1994a; Yap *et al*., 1994b; Boussiba *et al*., 2000; Armengol *et al*., 2005; Otieno-Ayayo *et al*., 2008). The *Anabaena* sp. produced the highest toxicity, with an LC_{50} value of 0.9 x 10^5 cells/ml (Wu *et al*., 1997; Boussiba *et al*., 2000).

1.5.6 Recombinant *Anabaena* sp. strain PCC 7120#11

Anabaena sp. have been considered as candidates for heterogeneously expressing *Bti* toxins as these organisms have several criteria that make them suitable candidates for genetic modification (Stevens *et al*., 1994; Manasherob *et al*., 2002; Manasherob *et al*., 2003). *Anabaena* sp. are widely distributed in natural waters worldwide and in lentic and lotic water bodies in South Africa (Harding & Paxton, 2001), they have limited nutritional

requirements (photoautotrophic mode of metabolism), capable of resisting a range of environmental conditions, and most species can float in the upper layer of the water bodies where the mosquitoes prefer to feed (Thiery *et al*., 1991; Murphy & Stephens, 1992; Tandeau de Marsac & Houmard, 1993; Stevens *et al*., 1994; Boussiba *et al.*, 2000; Manasherob *et al*., 2002).

Anabaena sp. strain PCC 7120 (hereafter referred to as PCC 7120), a filamentous, nitrogen-fixing cyanobacterium, is a laboratory strain that has its complete genomic sequence determined (Kaneko *et al*., 2001). Unlike other species of *Anabaena*, PCC 7120 does not produce neuromuscular toxins (Kurmayer & Jüttner, 1999). Before transferring any *Bti* genes into PCC 7120, several preliminary studies were performed in *Escherichia coli* (Ben-Dov *et al*., 1995). Three genes (*cry4Aa* and *cry11Aa* and the gene for the 20 kDa accessory protein, *p20*) were isolated and cloned in all seven possible combinations into two expression vectors (pT7 and pUHE), with *E. coli* as a host (Ben-Dov *et al*., 1995). The P20 protein is encoded by the third gene of the *cry11Aa* operon (Dervyn *et al*., 1995), and raises the expression levels of Cyt1Aa, Cry11Aa and Cry4Aa in *E. coli* and in an acrystalliferous strain of *B. thuringiensis*, acts as a chaperonin for stabilizing the gene products (Adams *et al*., 1989; Wu & Federici, 1993; Manasherob *et al*., 2001). P20 protected the *E. coli* cells from the lethal effect of Cyt1Aa (Manasherob *et al*., 2001). In addition, larvicidal activity was increased when *cry11Aa* was expressed together with the *p20* gene (Ben-Dov *et al*., 1995). The four combinations containing *cry4Aa*; *cry4Aa* and *cry11Aa*; *cry4Aa* and *p20*; and *cry4Aa*, *cry11Aa* and *p20* genes, all displayed high levels of larvicidal activity in pUHE (Ben-Dov *et al*., 1995).

The *cry4Aa*, *cry11Aa*, and *p20* genes were introduced into PCC 7120 using an *E. coli-Anabaena* shuttle vector pRL488p (Ben-Dov *et al*., 1995; Wu *et al*., 1997). They were expressed under the control of two tandem promoters, a cyanobacterial promoter (P_{psbA}) and an *E. coli* T7 promoter (P_{A1}). Two

independent pSBJ2 clones carrying pHE4-ADR (clone 11 and clone 7) were found to exhibit the highest larvicidal activity ever recorded for transgenic cyanobacteria against third instar *A. aegypti* larvae (Wu *et al*., 1997). Clone 11 had the *Bti* genes integrated into the chromosome of PCC 7120, whereas the second clone, clone 7 had the *Bti* genes as plasmid-borne constructs (Wu *et al*., 1997). Khasdan *et al*. (2003) introduced genes encoding the *Bti* toxin genes, *cry4Aa*, *cry11Aa*, *cyt1Aa*, and the accessory *p20* genes, into PCC 7120 resulting in the recombinant clone, pRVE4-ADRC. Although pRVE4-ADRC exhibited high larvicidal activity against fourth instar *A. aegypti* larvae (Khasdan *et al*., 2003), pRVE4-ADRC lost its larvicidal activity after four subcultures, suggesting it was plasmid-borne like clone 7 (Lluisma *et al*., 2001; Khasdan *et al*., 2003). The plasmid is lost in the absence of antibiotic selection, making pRVE4-ADRC and clone 7 unsuitable for biological control in the field (Khasdan *et al*., 2003). The recombinant clone, clone 11, was shown to be stable as the modified genes had integrated into the chromosome of PCC 7120 (Lluisma *et al*., 2001).

Anabaena sp. PCC 7120 clone #11 (hereafter referred to as PCC 7120#11) was constructed with the *Bti* toxins, *cry4Aa* and *cry11Aa*, together with a accessory protein, P20 (Wu *et al*., 1997). The toxicity to mosquito larvae was found to be higher than the commercial preparations of *Bti*, due in part to its ability to multiply in the mosquito breeding sites, maintain its position in the water column due to the presence of gas vacuoles, and resist inactivation by UV (Murphy & Stephens, 1992; Stevens *et al*., 1994; Boussiba *et al*., 2000; Manasherob *et al*., 2002; Khasdan *et al*., 2003). Before any potential biological control agent can be fully developed as a control agent, field trials need to be performed (Hershey *et al*., 1998; Crickmore, 2006). However, there are many concerns with testing or releasing a live, genetically engineered microorganism (GEM), like PCC 7120#11 into the environment (Levidow, 1995; Giddings, 1998). Some of the major concerns with regards to

releasing a GEM into the environment need to be addressed in the laboratory before any field trials can be performed (Crickmore, 2006).

1.6 Evaluation of GEMs in the laboratory

Researchers are concerned with the risks associated with releasing live GEMs into the environment (Williamson, 1992; Porter *et al*., 1993; Giddings, 1998; Stephenson & Warnes, 1996; Kaur, 2000). These concerns include gene transfer into other organisms, the unknown effects on beneficial non-target organisms, and unknown effects on biodiversity (Kimball & Levin, 1985; Porter, 1996; Stephenson & Warnes, 1996; Giddings, 1998; Boussiba *et al*., 2000; Wolfenbarger & Phifer, 2000). Potential environmental consequences of GEMs need to be considered before they can be released or commercialized (Kaur, 2000; Hynes & Boyetchko, 2006). Before any field trials using GEMs can commence, risk assessments have to be submitted to the regulatory authorities (Hynes & Boyetchko, 2006). Obtaining permission for release of GEMs into the environment is difficult (Stephenson & Warnes, 1996; Phillips, 2001), thus in order to reduce some of the concerns with releasing GEMs into the environment, regulatory agencies recommend the use of microcosms for environmental risk assessment of biopesticides like PCC 7210#11 (Crane, 1997). Microcosms can aid in testing the environmental safety of a GEM, determine its persistence in the environment, its toxicity to the environment, and save time and money for industry and regulatory agencies (Jepson *et al*., 1994; Krimsky *et al*., 1995).

Microcosms can be divided into two types: those that replicate the natural environment as much as possible and those that evaluate specific interactions between a chemical or microorganism and some environmental parameter (Krimsky *et al*., 1995). Synthetic microcosms are systems that mimic the conditions of the environment as much as is possible (Krimsky *et al*., 1995).

Environmental risk assessment of GEMs is largely based on the effects of single compounds in single-species test (Cuppen *et al*., 2002; Duchet *et al*., 2008). Single-species tests select the most sensitive species as they should indicate toxic effects and then assumes that all other species will thus be unharmed (Cairns, 1983; Flum & Shannon, 1987). The simplicity of single-species tests makes the data easier to interpret and also provides valuable information on the stability of a GEM as it is isolated from other species (Taub, 1992). However, single-species tests do not accurately predict the interactions of biotic or abiotic materials in an environment, or address processes such as competition and predation (Cairns, 1983; Kimball & Levin, 1985; Balczon & Pratt, 1994; Crane, 1997; Swartzmann *et al*., 1990). Thus, one cannot extrapolate the data from single-species laboratory tests to field situations (Cairns, 1983; Kimball & Levin, 1985). As a consequence, several researchers use multi-species tests which can range from simple indoor tests to field studies (Balczon & Pratt, 1994; Traunspurger *et al*., 1996).

Multi-species with multi-trophic level communities allow assessment of GEMs on numerous species of primary producers, grazers and decomposers, simultaneously exposed to the same conditions (Taub, 1992; Clément & Cadier, 1998). Laboratory multi-species microcosm tests can simulate field conditions to a degree and can be standardized and reproduced (Taub *et al*., 1986; Conquest & Taub, 1989). Multi-species tests that are easy to construct, replicate and maintain, and exhibit many ecosystem properties, are favourable (Cairns, 1983).

For the purposes of this thesis, a multi-species microcosm was evaluated, in order to determine the effect of PCC 7120#11 on an aquatic community. Two main types of microcosms that could be used to evaluate PCC 7120#11 are the Mixed Flask Culture (MFC) microcosms and the Standardized Aquatic Microcosm (SAM) (Flum & Shannon, 1987; Taub, 1992).

1.7 The MFC microcosm and the SAM protocol

The MFC microcosms consist of small, mixed flask cultures (1-litre beakers) containing synthetic medium and inoculum consisting of numerous algae, zooplankton, ostracods, amphipods, and other microorganisms (Flum & Shannon, 1987; Taub, 1989; Anderson & Shannon, 1992). The inoculum, originally collected from the field, is allowed to develop for at least three months in a synthetic medium (Taub & Dollar, 1964; Flum & Shannon, 1987; Anderson & Shannon, 1992). The focus of MFC microcosms is on common ecosystems processes and not on the species involved in the processes, however MFC microcosms should contain a mixture of photosynthetic microorganisms (PMOs) which include algae and cyanobacteria, one species of herbivorous grazer, one species of benthic detritivore, protozoa, and associated bacteria (Flum & Shannon, 1987; Yount & Shannon, 1987; Taub, 1989; Taub, 1992).

In contrast, a SAM experiment consists of the same species (10 PMOs and 5 invertebrates) in similar numbers and culture state (Taub *et al*., 1988; Taub, 1989; Taub, 1992; Taub, 1997). The protocol is standardized in that each experiment starts with the same chemically defined medium and sediment, same concentration of species, and same species (Taub *et al*., 1988; Taub, 1989; Taub, 1993). The idea of standardizing microcosm studies is that it makes comparison of data from diverse microcosms possible and allows for replication of results (Krimsky *et al*., 1995; Taub, 1997). The SAM is a small, simple and reliable system that mimics a generalized aquatic ecosystem (Taub, 1989; Meador *et al*., 1993; Taub, 1993). The selection of organisms used in the SAM experiment was based on several factors such as ease of culturing the organisms in the laboratory, organisms that are typically used in single-species tests, and organisms that could grow in a single medium (Taub, 1989; Taub, 1993). The PMO community was designed to have greens, blue-greens, and diatoms; and the main invertebrate populations usually found in freshwater such as protozoans, rotifers, copepods, and

cladocerans, were added to the SAM experiment (Taub, 1989; Sterner & Hessen, 1994).

Although the SAM protocol is more labour intensive than the MFC protocol, it is more repeatable, more reproducible, and provided more precise taxonomic distinctions (Taub, 1989; Taub, 1992). Thus, the SAM protocol was used to evaluate the effects of PCC 7120#11 in an aquatic environment. The SAM protocol is more complex than the single-species tests but it is not as complex as a natural environment, thereby allowing the interactions within and between trophic levels to be evaluated (Taub, 1992; Taub, 1997). The SAM protocol is designed to demonstrate primary production, secondary production, and recycling, but with multiple species per trophic level (Taub, 1989). In a standardized system there are less taxonomic uncertainties, thus the focus is more on interpreting ecological relationships (Taub, 1997). Another favourable characteristic of the SAM experiment is the rapidity with which the experiments can be conducted, analyzed and, the microcosms can be initiated at any time of the year (Taub, 1997). The individual components that make up the microcosm are discussed below.

1.7.1 Sediment

In addition to sand, the sediment contains chitin and cellulose as a form of detritus (Taub, 1989). Sediment is a key environmental component as it acts as a sink for many toxins (Clément & Cadier, 1998).

1.7.2 Nutrients available in the microcosms

The medium known as T82MV (Taub, 1989), was designed to meet the nutritional requirements of the PMOs and other organisms. It is a medium that is capable of supporting the growth of many freshwater PMOs and is non-toxic to *Daphnia* sp. (Taub, 1989). In the SAM experiment, the main inorganic nutrients (ammonia, nitrate, nitrite, and phosphate) are supplied in the media (Taub, 1989; Taub, 1993).

1.7.3 Primary producers

Primary producers are organisms that are capable of converting inorganic chemicals and energy into organic compounds (Wiegert & Owen, 1971). In this ecosystem the primary producers are the PMOs such as algae and cyanobacteria, *Anabaena cylindrical*, *Ankistrodesmus* sp., *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Oscillatoria simplicissima*, *Scenedesmus obliquus*, *Selenastrum capricornutum*, *Nitzschia* sp., *Stigeoclonium* sp., and *Ulothrix* sp. The PMO species were chosen to represent the major classes of PMOs (e.g. unicellular green algae, filamentous cyanobacteria, and diatoms) (Taub, 1989). *A. cylindrical* is a filamentous cyanobacterium that has potential to fix nitrogen. *C. reinhardtii*, *C. vulgaris*, *S. capricornutum*, *S. obliquus,* and *Ankistrodesmus* sp. are all green unicellular algae, but *S. obliquus* tends to form clusters of four (Taub, 1989). *Stigeoclonium* sp. and *Ulothrix* sp. are both green filamentous algae that are generally utilized by amphipods (Taub, 1989). *Nitzschia* sp. is a diatom and *O. simplicissima* is a filamentous cyanobacterium that does not fix nitrogen (Taub, 1989). PMO biomass consists mainly of carbohydrates, proteins, lipids and can contain all the elements essential for herbivore nutrition (Sterner & Hessen, 1994).

1.7.4 Secondary producers

Secondary producers or grazers are organisms that require organic chemicals for their energy source (Sterner, 1993). All the invertebrates in the microcosms were secondary producers (Taub, 1989). The secondary producers generally used in the SAM are ostracods (*Cyprinotus*), cladocerans (*Daphnia magna*), rotifers (*Philodina* sp.), protozoans (Hypotrichs), and amphipods (*Hyallela azteca*) (Taub, 1989).

1.7.4.1 Cladocerans

Most of the world's 400 or so species of Cladocera are found in freshwater (Seaman *et al*., 1999). The family Daphniidae contain the best-known cladocerans, with most of the southern African species of *Daphnia* sp.

commonly found in ponds or temporary waters (Seaman *et al*., 1999). Cladocerans vary in length from <1 mm to nearly 5 mm (Gilbert, 1988). Most are filter-feeders, ingesting unicellular algae and various sorts of organic detritus including protists and bacteria (Forró *et al*., 2008). *Daphnia pulex* was evaluated in our study.

1.7.4.2 Ostracods

Ostracods, found in the zoobenthos in African inland waters, are relatively unselective scavengers and form an important part of aquatic ecosystems (Diner *et al*., 1986; Martens, 2001). Ostracods are small to medium sized bivalved crustaceans, with eight larval instars and the ninth instar being the adult (Martens, 2001). The first three instars generally do not feed and only start to feed actively from the fourth larval instar (Martens, 2001). The Cyprididae are the most successful ostracod group in Africa, with 80% of all African non-marine ostracods species being cypridids (Martens, 1998). The species used in this study is *Cyprinotus africanus*.

1.7.4.3 Protozoans

Protozoans are single-celled eukaryotes, found in all natural and most man-made freshwater habitats (Hegg, 2002). Their nutrition can be autotrophic, heterotrophic or both, with bacteria being an important food source for heterotrophic protozoans (Hegg, 2002). Protozoans play an important role in the decomposition of organic matter in aquatic environments (Legner, 1973).

Protozoans reproduce asexually by means of binary fission, thereby multiplying rapidly under favourable conditions (Hegg, 2002). Several environmental factors have been identified as determining the composition of the protozoan community in any particular water body with swamps, ponds and lakes, supporting the greatest diversity of protozoans (Hegg, 2002). The protozoans recommended for use in the SAM experiment belong to the order Hypotrichida, which consists of ciliates that are generally found in vegetation

and bottom debris (Hegg, 2002). However, the order Gymnostomatida was used in this study.

1.7.4.4 Rotifers

Rotifers have an important place among microscopic freshwater invertebrates, as they feed on bacteria and detritus and have a rapid turnover, thereby playing an important part in the production cycle (Brain, 2002). The phylum Rotifera contains the classes, the Digononta and the Monogononta (Brain, 2002). The Monogononta, make up 90% of known species, have single ovaries and the males are typically small and degenerate (Brain, 2002). Of interest to us are the rotifers, *Lepadella* sp. belonging to the Monogononta class in the order Colurellidae (Brain, 2002).

1.7.4.5 Amphipods

The order Amphipoda contains over 6000 species worldwide with two freshwater suborders, the Gammaridea and Ingolfiellidea, with the Grammaridea incorporating about 80% of all known amphipod species (Griffiths & Stewart, 2001). The southern African freshwater gammarid fauna are found in streams and sometimes caves in the western and southern Cape and restricted to caves in KwaZulu Natal and Northern Cape (Griffiths & Stewart, 2001). These freshwater gammarids are not likely to be found in water bodies that malaria vectors tend to breed, hence they were not included in the microcosm studies.

1.8 Large scale production of genetically engineered cyanobacteria

Provided that PCC 7120#11 does not pose a threat to non-target organisms and to the environment, PCC 7120#11 could be used as a potential control larvicidal agent. If field trials or field applications are to be performed, large quantities of PCC 7120#11 will be required. Thus, optimal production of PCC 7120#11 was investigated.

Photobioreactors have been used for several years for the mass production of PMOs for human and animal nutrition (Benemann *et al*., 1987; Borowitzka, 1997; Brown *et al*., 1999; Soletto *et al*., 2005; Spolaore *et al*., 2006), biofertilizers (Boussiba, 1991; Sinha & Häder, 1996), pharmaceuticals (Benemann *et al*., 1987; Belay *et al*., 1993), exopolysaccharides (Moreno *et al*., 1998), renewable energy, and biofuel production (Tsygankov *et al*., 2002; Yoon *et al*., 2002; Pulz & Gross, 2004; Yoon *et al*., 2006). Studies currently focus on using photobioreactors to improve or increase PMO production to maximum efficiency (Richmond & Cheng-Wu, 2001).

Although there are several advantages to culturing cyanobacteria in photobioreactors, there are many obstacles researchers have to overcome such as reducing contamination, providing sufficient light and carbon dioxide, controlling culture conditions like pH and temperature, and reducing production costs (Grobbelaar *et al*., 1996; Richmond *et al*., 2003; Posten, 2009). Two major systems have been used for the cultivation of cyanobacteria; the open cultivation system and the closed cultivation system.

1.8.1 Open cultivation systems

Different types of open cultivation systems have been utilized, with the most commonly used systems being shallow big ponds, tanks, circular ponds, and raceway ponds (Borowitzka, 1999; Pulz, 2001; Xu *et al*., 2009).

Although some of the advantages of open cultivation systems are that they have direct access to sunlight, and they are easier to build and operate than closed cultivation systems, a number of major drawbacks have been identified with using open systems. Some of the disadvantages of open cultivation systems include; only certain cyanobacterial species are suited for open large scale cultivation, contamination risks from other microorganisms, evaporation, loss of carbon dioxide, space requirements, biomass productivity is lower than that in closed cultivation systems, monitoring and controlling environmental

conditions, and harvesting costs are high (Iqbal *et al*., 1993; Hu & Richmond, 1996; Borowitzka, 1999; Pulz, 2001; Xu *et al*., 2009). As a result of these limitations, focus has shifted to the development of closed cultivation systems.

1.8.2 Photobioreactor design properties

Biotechnologists have to consider several important factors when designing photobioreactors for closed system cultivations such as illuminance, light path, turbulence, aeration, and carbon dioxide supply (Ugwu *et al*., 2008; Posten, 2009; Xu *et al*., 2009).

Provided temperature and nutrients do not limit growth, light is the main limiting factor of growth in photobioreactors (Lee *et al*., 1996; Tredici & Zittelli, 1998; Ogbonna & Tanaka, 2000; Richmond, 2000). Photobioreactors use either natural or artificial illumination (Muller-Feuga, 1998; Xu *et al*., 2009). PMOs contain photosynthetic receptor systems, photosystem I and photosystem II reaction centres, for capturing light energy from the light harvesting complexes (Cruz *et al*., 2005). Illuminance above a certain threshold (approximately 32000 lux) can damage the photosystem II reaction centres (Camacho *et al*., 1999; Pulz, 2001; Molina *et al*., 2000). This phenomenon, known as photoinhibition, usually results in the formation of toxic products such as H_2O_2, O_2^-, and OH (Chojnacka & Noworyta, 2004). However, if the illuminance is low, photosynthesis may be limited thereby reducing biomass productivity (Gordon & Polle, 2007; Jacob-Lopes *et al*., 2008). Illuminance can be supplied continuously or in cycles of light-dark (Richmond *et al*., 2003). Light-dark cycles play an important role in the photosynthetic activity of the cells in the photobioreactor whereby the cells are exposed to intermittent periods (millisecond flashes) of darkness (at a specified frequency) which leads to increased light utilization (Benemann *et al*., 1987; Grobbelaar, 1994; Hu *et al*., 1998a; Camacho *et al*., 1999; Grobbelaar, 2000). The level of sensitivity to illuminance varies with the

species evaluated (Chisti, 2007), as some species can adapt to different illuminance (Zhang *et al*., 2001b; Posten, 2009).

When the cell density of the culture in the photobioreactor increases, the light transmitted through a culture decreases, thus the length of the light-path of the photobioreactor is also crucial in improving the light to which the cells are exposed (Hu *et al*., 1996; Hu *et al*., 1998a; Zou & Richmond, 1999). High biomass concentrations can be obtained in short light-path flat panels, as a result of a shorter distance from the light source to the cultures providing sufficient illuminance for the cells in photobioreactor (Hu *et al*., 1998a; Zou & Richmond, 1999; Richmond, 2000; Yoon *et al*., 2008). Although a short light-path is favourable to improving volumetric output, it must be defined for each algal species (Zou & Richmond, 1999; Richmond *et al*., 2003).

Turbulence in the photobioreactors ensures optimal illuminance by carrying the cells from the well illuminated zones to the poorly illuminated zones, thereby increasing biomass productivity (Hu & Richmond, 1996; Hu *et al*., 1996). Turbulence also aids in circulation of nutrients and mass transfer of gases in the photobioreactor (Fontes *et al*., 1987; Grobbelaar, 1994; Grobbelaar *et al*., 1996; Lehr & Posten, 2009). However, the rate of turbulence is restricted because some PMO species cannot deal with the high shear stress caused by high turbulence (Gudin & Chaumont, 1991; Hu & Richmond, 1996; Converti *et al*., 2006).

PMOs utilize carbon dioxide as a carbon source (Yoon *et al*., 2002), however excess carbon dioxide can be inhibitory to the cells (Soletto *et al*., 2008). Excess oxygen is also inhibitory to growth of the cells as it causes photobleaching and is inhibitory to photosynthesis (Sierra *et al*., 2008; Dasgupta *et al*., 2010). Thus, optimal growth of the PMOs in photobioreactors depends on a good mass transfer of carbon dioxide and oxygen (Lehr & Posten, 2009).

1.8.3 Closed cultivation systems

Closed photobioreactors allow for better control of cultivation conditions and for reduced contamination risks than open cultivation systems (Ugwu *et al*., 2008). Various designs of photobioreactors have been proposed and investigated for the mass production of cyanobacteria, with the main photobioreactor designs for closed cultivation systems including tubular, flat-plate, airlift and bubble-columns (Torzillo *et al*., 1986; Lee *et al*., 1995; Richmond *et al*., 1993; Pulz, 2001; Zhang *et al*., 2001a; Zhang *et al*., 2001b).

1.8.3.1 Tubular photobioreactors

Tubular photobioreactors including helical and horizontal tubular photobioreactors, are simple and easy to construct, however they are expensive for PMO mass cultivation (Richmond *et al*., 1993; Watanabe & Hall, 1995; Mirón *et al*., 1999; Molina *et al*., 2001; Ugwu *et al*., 2002; Ugwu *et al*., 2005). Tubular photobioreactors consist of an array of straight, coiled or looped transparent plastic or glass tubes (Mirón *et al*., 1999; Fernandez *et al*., 2001). Pump or airlift technology moves the PMOs through the tubes (Molina *et al*., 2000). The length and diameter of the tubes is important in tubular photobioreactors (Molina *et al*., 2000). If the tubes are too long, oxygen accumulates and could lead to inhibition of photosynthesis (Molina *et al*., 2000). If tube diameter is greater than 0.1 m, as the cultures increase in concentration, self-shading of the cells occurs causing a decrease in volumetric output (Xu *et al*., 2009).

1.8.3.2 Airlift and bubble-column photobioreactors

Airlift and bubble-column photobioreactors are simple, compact, vertical columns used in bioprocessing, wastewater treatment, aquaculture, and the chemical process industry (Xu *et al*., 2009; Chisti & Moo-Young, 1993; Mirón *et al*., 2002). The advantages of using airlift and bubble-column photobioreactors is that there is high gas mass transfer rate, rapid mixing, low physical stress, and low energy costs (Chisti, 1998; Mirón *et al*., 1999;

Camacho *et al*., 1999). As in the tubular photobioreactors, the diameter of the column is a limiting factor, making scale up of airlift and bubble column reactors difficult (Mirón *et al*., 1999; Xu *et al*., 2009).

1.8.3.3 Flat-plate photobioreactors

Flat-plate photobioreactors were first described by Samson and Leduy (1985), and were developed further by Tredici *et al*. (1991). The photobioreactor, initially made from transparent polycarbonate sheets partitioned to form rigid narrow alveolar channels (Tredici *et al*., 1991), was tilted to provide maximum exposure of the culture to illuminance (Hu *et al*., 1996; Hu *et al*., 1998b). The flat, inclined modular photobioreactor (FIMP) was designed so that the light path of the flat-plate could be easily manipulated (Hu *et al*., 1996; Hu *et al*., 1998b). In vertical flat-plate photobioreactors, high volumetric output concentrations can be reached in the narrow, light-path flat panels (Zhang *et al*., 2001a).

Flat-plate photobioreactors have a high surface to volume ratio and design of the photobioreactor is suitable for a short light-path (Hu *et al*., 1996; Hu *et al*., 1998b). Mixing in the flat-plate photobioreactors involves introducing air or a mixture of carbon dioxide and air via a perforated tube at the bottom of the reactor, reducing shear stress (Hu *et al*., 1998b; Xu *et al*., 2009). The flat-plate photobioreactors are usually illuminated on one side and they can be positioned vertically, horizontal or inclined, at an optimum angle facing the light source (sun or artificial light) (Hu *et al*., 1998b). The advantage of using an inclined photobioreactor is that illuminance entering the photobioreactor can be controlled and absorption of incident energy is higher (Hu *et al*., 1998a; Hu *et al*., 1998b). For the purposes of this thesis, an indoor, flat-plate inclined photobioreactor was designed and used to optimise the growth of PCC 7120#11.

1.9 Justification of the study

Currently, most of the mosquito control methods being used are not ecologically friendly and pose a threat to the environment and non-target organisms (Gratz & Pal, 1988; Rose, 2001; Federici *et al*., 2003; Stevens *et al*., 1994). There are also reports of development of resistance to the chemical control agents currently being used (Rose, 2001; Ranson *et al*., 2001; Hougard *et al*., 2002; Shiff, 2002; Coetzee, 2004). Thus, there is a need to identify and evaluate alternative mosquitocidal control agents. Research has shown that a recombinant *Anabaena* PCC 7120 (*Anabaena* PCC 7120#11), which carries specific *Bti* toxin genes, is a very effective mosquitocidal agent in laboratory bioassays (Wu *et al*., 1997; Lluisma *et al*., 2001; Manasherob *et al*., 2003), suggesting that it may have potential as a control agent of malaria mosquito vectors in southern Africa.

To determine the potential of PCC 7120#11 as a biological control agent of southern African malaria vectors, it is necessary to compare it against the most widely used mosquito biological control agent, *Bti*. To our knowledge, no studies have evaluated comprehensively, under the same experimental conditions and using the same *Bti* preparation, the larvicidal activity of *Bti* against the most important African anopheline species. The larvicidal activity of *Bti* against each anopheline species would provide a point of reference to compare against the larvicidal activity of PCC 7120#11. In addition to evaluating the larvicidal activity of PCC 7120#11, the environmental safety of PCC 7120#11 has to be evaluated, as there may be several potential risks (e.g. detrimental effects on non-target organisms and water quality) associated with releasing a genetically engineered cyanobacterium into the environment. Evaluation of the effects of PCC 7120#11 and *Bti* on non-target invertebrates and on water quality would assist in determining if PCC 7120#11 may be a more environmentally friendly control agent than *Bti*. The use of standardized aquatic microcosms for the evaluation of the effects on non-target organisms and water quality would allow for comparison of data from different microcosms and enable replication of results. Since large-scale production of a biological control agent is necessary if a control agent is to be

used in a vector control program, the large-scale production of PCC 7120#11 has to be evaluated and optimized as a precursor to potential commercialization of PCC 7120#11. This thesis evaluated the potential of PCC 7120#11 as a biological control agent of southern African malaria vectors through evaluation of the above criteria.

1.10 Aims and objectives

The main objective of this study was to evaluate two *Bti* based biopesticides, PCC 7120#11 and *Bti*, for their potential to control southern African malaria vectors.

The specific aims of the study were:

1. To evaluate the larvicidal activity of *Bti* against larvae of key *Anopheles* species by laboratory bioassays.

2. To evaluate the larvicidal activity of PCC 7120#11 against larvae of key *Anopheles* species by laboratory bioassays.

3. To evaluate the effects of *Bti* on target and non-target organisms in standardized aquatic microcosms.

4. To evaluate the effects of PCC 7120#11 on non-target organisms and the larvicidal persistence of PCC 7120#11 in aquatic microcosms.

5. To determine optimal growing conditions of PCC 7120#11 in an indoor, flat-plate inclined photobioreactor.

Chapter 2

The larvicidal activity of *Bacillus thuringiensis* subspecies *israelensis* against five African *Anopheles* (Diptera: Culicidae) species

2.1 Abstract

There are an estimated 300-500 million cases of malaria worldwide and over 1.2 million malaria-related deaths reported each year. Biological control programs using microbial agents have proven not only to be effective against a wide variety of mosquito species but are also environmentally safe, and target specific. *Bacillus thuringiensis* (*Bt*) is toxic to a wide variety of insect larvae, mostly lepidopteran, coleopteran, and dipteran species. The objective of this study was to examine the larvicidal activity of *Bacillus thuringiensis* subspecies *israelensis* (*Bti*) strain HD-522 against major African malaria-carrying vectors, including the key malaria vectors *Anopheles arabiensis*, *Anopheles funestus*, and *Anopheles gambiae*. All the anopheline species evaluated in this study were susceptible to *Bti* spore-crystal suspensions, although there were differences in the susceptibility of the species to *Bti*. The LC_{50} values did not differ significantly between the vectors *Anopheles merus, An. gambiae*, *An. arabiensis*, and *An. funestus*. *Anopheles quadriannulatus* was the most susceptible to *Bti*, with its LC_{50} value of 2.97×10^4 spores/ml being significantly lower than that of the other anopheline mosquitoes evaluated. The susceptibility order of the anopheline species was different when it was based on the LC_{90} values, with the LC_{90} of *An. arabiensis* being significantly higher than that of *An. quadriannulatus* or *An. merus.* Different feeding rates and behaviour may affect susceptibility of the anopheline species to *Bti*. Concentration-mortality regression slopes indicated that *An. quadriannulatus* and *An. merus* exhibited lower population response variability to *Bti* than the other vectors evaluated. This study provided valuable information for vector control programs by showing which of the anopheline species are more susceptible to *Bti* and the *Bti* concentration required for effective vector control.

2.2 Introduction

Malaria is caused by protozoan parasites of the genus *Plasmodium,* with most deaths being caused by *Plasmodium falciparum* (Phillips, 2001; Cowman & Crabb, 2006). There are an estimated 300-500 million cases of malaria worldwide and over 1.2 million malaria-associated deaths reported each year, mostly in young children in sub-Saharan Africa (Breman, 2001; WHO, 2008; Murray *et al*., 2012). The direct and indirect costs of malaria in Africa are estimated to exceed 2 billion U.S dollars per year (Kager, 2002; Sachs & Malaney, 2002), thus it is of utmost importance to control the disease and more importantly, the spread of the disease.

Mosquitoes in the genus *Anopheles* are the most efficient vectors of malaria worldwide. Two important groups within the genus *Anopheles* that play a major role in malaria transmission in Africa, are the *Anopheles gambiae* Giles complex and the *Anopheles funestus* Giles group (Koekemoer *et al*., 2002). The *An. gambiae* complex comprises seven named species, one unnamed species, and several “incipient” species (Coetzee *et al*., 2000; Coetzee, 2004; Moreno *et al*., 2007). The seven named species are *Anopheles gambiae* Giles *sensu stricto*, *Anopheles arabiensis* Patton, *Anopheles quadriannulatus* Theobald (species A and B), *Anopheles bwambae* White, *Anopheles merus* Dönitz, and *Anopheles melas* Theobald (Gillies & Coetzee, 1987; Hunt *et al*., 1998; Coetzee *et al*., 2000). Members of the *An. gambiae* complex are widely distributed and have been reported in most countries of Africa and its adjacent islands, including Madagascar (Coetzee *et al*., 2000). Most of the species in the group breed in freshwater, but *An. merus* and *An. melas* are salt-water breeders while *An. bwambae* is found in mineral hot springs (Gillies & Coetzee, 1987; Coetzee *et al*., 2000). The species in the *An. gambiae* complex contain excellent vectors of malaria (*An. gambiae* and *An. arabiensis*) as well as non-vectors (*An. quadriannulatus*) (Coetzee *et al*., 2000).

An. gambiae, one of the most efficient malaria vectors in the world, is widespread in tropical Africa (Gillies & De Meillon, 1968). It is highly anthropophilic and endophilic (Gillies & De Meillon, 1968). *An. arabiensis* is the most widespread vector of malaria in tropical and southern Africa (Hargreaves *et al*., 2003; Coetzee, 2004). The females exhibit variable behaviour being both anthropophilic and zoophilic, as well as endophilic and exophilic (White, 1974; Gillies & Coetzee, 1987). As a result of the variable behaviour of *An. arabiensis*, the efficiency of some of the current vector control methods are reduced (Collins & Besansky, 1994).

The *An. funestus* group comprises nine species, these being *Anopheles funestus* Giles, *Anopheles parensis* Gillies, *Anopheles rivulorum* Leeson, *Anopheles vaneedeni* Gillies and Coetzee, *Anopheles leesoni* Evans, *Anopheles fuscivenosus* Leeson, *Anopheles aruni* Sobti, *Anopheles brucei* Service, and *Anopheles confusus* Evans and Leeson (Koekemoer *et al*., 2002). *An. funestus* is the most important and efficient vector of malaria parasites in this group (Hargreaves *et al*., 2003; Coetzee & Fontenille, 2004). It is highly anthropophilic and endophilic (Coetzee & Fontenille, 2004). The other members of the group are mainly zoophilic, with only *An. rivulorum* considered to be a minor vector in Tanzania (Wilkes *et al*., 1996). *An. funestus* is widely distributed throughout Africa and is responsible for contributing to one of the most recent malaria epidemics in South Africa (Hargreaves *et al*., 2000; Hargreaves *et al*., 2003; Coetzee, 2006).

Integrated malaria vector control programs target both the larval and adult stages of the mosquitoes (Walker & Lynch, 2007; Beier *et al*., 2008). Many chemical insecticides such as pyrethroids, organochlorines, organophosphates, and carbamates are used in the control of the malaria vectors (Phillips, 2001; Shiff, 2002; Okoye *et al*., 2008). Adult control programs use indoor residual insecticide spraying or insecticide-treated fabrics such as bed nets and curtains (Coosemans & Carnevale, 1995;

N'Guessan *et al*., 2007; Okoye *et al*., 2008). Larval control programs rely on adequate information about the habitats, distribution, and behaviour of the larval vector (Rozendaal, 1997; Walker & Lynch, 2007). Removal or reduction of the larval habitats as well as treating the mosquito breeding sites with larvicidal agents are crucial to larval control (WHO, 1995). A major problem with adult and larval control programs is the development of resistance by the vectors to the chemical insecticides (Ranson *et al*., 2001; Rose, 2001; Hougard *et al*., 2002; Shiff, 2002; Coetzee, 2004). The important malaria vector species, such as *An. funestus, An. gambiae*, and *An. arabiensis*, have demonstrated resistance to insecticides such as carbamates, pyrethroids, and dichlorodiphenyltrichloroethane (DDT) (Hargreaves *et al*., 2000; Hargreaves *et al*., 2003; Okoye *et al*., 2008; Awolola *et al*., 2009; Mouatcho *et al*., 2009; Vezenegho *et al*., 2009). Another disadvantage of current chemical control programs is the detrimental effects on the environment and non-target organisms (Gratz & Pal, 1988; Rose, 2001; Federici *et al*., 2003).

Biological control offers an environmentally friendly strategy for the control of malaria vectors (Takken & Knols, 2008). Biological agents used to control malaria vectors include fungi, nematodes, fish, and bacteria (Petersen & Willis, 1974; Davidson, 1982; Homski *et al*., 1994; Cuda *et al*., 1997; Pérez-Pacheco *et al*., 2005; Scholte *et al*., 2005; Crickmore, 2006). Biological control programs using microbial agents have proven not only to be effective against a wide variety of mosquito species (Becker & Rettich, 1994; Lacey *et al*., 2001; Fillinger *et al*., 2003; Pérez-Pacheco *et al*., 2005; Scholte *et al*., 2005), but are also environmentally safe, and target specific (Das & Dominic Amalraj, 1997; Wirth *et al*., 1997; Fourcy *et al*., 2002). *Bacillus thuringiensis* subspecies *israelensis* (*Bti*) is an aerobic, spore-forming, Gram-positive bacterium that produces proteinaceous, crystalline inclusions during sporulation (Höfte & Whiteley, 1989; Schnepf *et al*., 1998). The insecticidal activity of *Bti* is due to the crystal (Cry) and cytolytic (Cyt) proteins found in the inclusions (Ibarra & Federici, 1986; Federici *et al*., 2003). The inclusions are

composed of at least four major proteins; Cyt1Aa (27 kilodalton (kDa)), Cry11Aa (72 kDa), Cry4Aa (128 kDa), and Cry4Ba (134 kDa) (Ibarra & Federici, 1986; Federici *et al*., 2003). These proteins (protoxins) are known to be toxic to mosquito larvae but have weak activity against non-Dipteran insects (Höfte & Whiteley, 1989).

The larvicidal activity of *Bti* strains and formulations against several mosquito species such as *Culex*, *Aedes* and *Anopheles* species, has been reported by several researchers (Majori & Ali, 1984; Klowden *et al*., 1983; Lacey & Inman, 1985; Majori *et al*., 1987; Wraight *et al*., 1987; Karch *et al*., 1991; Skovmand & Sanogo, 1999; Dominic Amalraj *et al*., 2000; Fillinger *et al*., 2003; Gunasekaran *et al*., 2004). However, no studies to our knowledge have examined the larvicidal activity of *Bti* against all the major African malaria vectors and it is thus important, from a potential control perspective, to evaluate the response of these anopheline species to *Bti.* In this study, we compared the larvicidal activity of *Bti* strain HD-522 against five anopheline species, including the key malaria vectors *An. arabiensis*, *An. funestus*, and *An. gambiae*.

2.3 Methodology

2.3.1 Culturing of *Bti*

Bti strain HD-522 was obtained from the Bacillus Genetic Stock Centre (BGSC; Ohio State University, Columbia, Ohio, U.S.A.). Strain HD-522 (OVR60) was selected because it has well characterised larvicidal activity against several species of mosquitoes (Goldberg & Margalit, 1977; Margalit & Dean, 1985). *Bti* was cultured in nutrient broth-yeast extract sporulation medium (NYSM) (Myers & Yousten, 1980) at 30 °C for 12 hours and a loopful of the starter culture was seeded into fresh NYSM medium, incubated at 30 °C at 240 revolutions per minute for 96 hours or until more than 95% sporulation was obtained. Sporulation was monitored by phase contrast microscopy.

2.3.2 Spore and crystal harvesting

The spores and crystals were harvested through differential centrifugation at 6000 *g* (Beckman J2-21 with JA-14 rotor, Beckman Coulter, U.K.) for 20 minutes at 4 °C. The supernatant was discarded and the pellet was washed by resuspension in sterile distilled water. The washing step was repeated thrice, using the same conditions as described above, and the resuspension pellet was stored at 4 °C.

2.3.3 Determination of spore concentration

Spore concentrations were determined by the pour plate method. A 10^{-2} dilution of the harvested spore-crystal suspension (SCS) was prepared (in triplicate), in sterile test tubes, with sterile distilled water and heated at 65 °C for 30 minutes, with agitation every 5 minutes. Serial dilutions of the cooled SCSs were prepared in duplicate and the pour plates were incubated at 30 °C for 12 hours before colonies were counted with a colony counter.

2.3.4 Dry weight determination

The dry weight of the SCS was determined as an additional method of quantification. Two hundred microlitres of the harvested SCS was transferred onto a pre-weighed aluminium weighing boat and heated at 90 °C for 24 hours. The boats were allowed to cool in a dessicator to prevent any absorption of moisture by the powder during the cooling period. The procedure was repeated three times and the average dry weight was determined.

2.3.5 Mosquito species

All the mosquito species (origin and colony name provided), *An. arabiensis* (Kanyemba, Zimbabwe; KGB), *An. funestus* (Maputo, Mozambique; FUMOZ), *An. merus* (KwaZulu Natal, South Africa; MAF), *An. quadriannulatus* species A (Sangwe, Zimbabwe; SANGWE), and *An. gambiae* (Ibadan, Nigeria; NAG) tested in this study were obtained from colonies maintained at the National

Institute of Communicable Diseases in Johannesburg, South Africa. The larvae were reared in a controlled environment of 25 °C, relative humidity ranging from 60-80%, and a L:D 12:12 hour photocycle with a 45 minute dawn and dusk light regime.

2.3.6 Laboratory bioassays

For each cup bioassay, 20 third instar mosquito larvae were selected, rinsed in sterile distilled water, and added to 98 ml of sterile distilled water in 130 ml white plastic cups. In the case of *An. merus*, a sterile 5 M saline solution was used instead of sterile distilled water. Dilutions of the SCS were prepared in sterile distilled water to obtain the appropriate range of *Bti* concentrations that would result in 5 to 95% larval mortality. Two millilitres of the appropriate SCS dilution was added to the respective plastic cup. The larvicidal activity was determined after 24 hours incubation at 25 °C at relative humidity of 60%, with a L:D 12:12 hour photocycle. Larvae were presumed dead if they did not move when prodded. Untreated controls were run for each dilution. If mortality in the controls exceeded 5% the test was discarded and repeated. Each bioassay was repeated in triplicate on three separate occasions. The median lethal concentration (LC_{50}) of each species was determined by probit analysis (Finney, 1971). For each species, probit analysis was based on the mortality data obtained from five SCS concentrations.

2.4 Results

Bti strain HD-522 produced a spore count of 6.57 x 10^9 spores/ml in the NYSM medium. The dry weight of the harvested SCS was determined to be 4.25 ng/µl (n = 3).

The heterogeneity factor defines the goodness of fit to the linearity of the concentration-mortality relationship (Champ & Campbell-Brown, 1970). The heterogeneity factor varied between the species examined, ranging from 0.80

to 0.11 (Table 2.1). The values are all lower than 1, indicating a good fit to the probit model.

The LC_{50} values did not differ significantly between the mosquitoes *An. gambiae*, *An. arabiensis*, and *An. merus* (Table 2.1). *An. funestus* had the highest LC_{50} value (4.44 x 10^4 spores/ml), but it was not significantly different to the above mentioned species (Table 2.1). The LC_{50} value of *An. quadriannulatus* was significantly lower than that of the other anopheline species evaluated, thus *An. quadriannulatus* was the most susceptible to *Bti* (Table 2.1). On comparison of the LC_{90} values, a different susceptibility order was observed with the LC_{90} of *An. arabiensis* being significantly higher than that of *An. quadriannulatus* or *An. merus* (Table 2.1). The LC_{90} of *An. funestus* was also significantly higher than that of *An. merus*, *An. gambiae* or *An. quadriannulatus* (Table 2.1).

Table 2.1 Probit analysis of concentration-mortality data for *Bacillus thuringiensis* subsp. *israelensis* against third instar larvae of African anopheline mosquitoes.

Species	LC_{50} (10^4 spores/ml) [1]	LC_{90} (10^4 spores/ml) [1]	Slope ± SE [2]	Heterogeneity [3]
An. quadriannulatus	2.97 (2.83 – 3.12)^a	5.02 (4.55 – 5.78)^a	5.64 ± 0.57^c	0.28
An. arabiensis	3.72 (3.38 – 4.10)^b	10.1 (8.51 – 12.7)^{cd}	2.96 ± 0.25^a	0.11
An. gambiae	3.76 (3.50 – 4.04)^b	7.70 (6.83 – 8.97)^{bc}	4.12 ± 0.32^b	0.43
An. merus	3.82 (3.62 – 4.03)^b	6.65 (6.08 – 7.54)^b	5.33 ± 0.49^c	0.72
An. funestus	4.44 (3.99 – 4.95)^b	13.5 (11.1 – 17.9)^d	2.65 ± 0.25^a	0.80

[1] Spore concentration in the assayed SCS. Values in brackets show the 95% fiducial limits (FLs). Values in a column followed by the same letters are not significantly different (non-overlapping 95% FLs).

[2] Slope ± standard error. Values followed by the same letters are not significantly different ($p > 0.05$).

[3] Heterogeneity factor = χ^2 / d.f. (degrees of freedom).

2.5 Discussion

The spore count of 6.57 x 10^9 spores/ml obtained in this study is slightly higher than the spore counts reported by other researchers (Obeta & Okafor,

1984; De Araújo *et al*., 2007). However, the concentration of viable spores from a culture varies according to several factors including temperature, pH, dissolved oxygen and nutrients available (Skovmand *et al*., 2000). The concentration of *Bti* spores is also not necessarily directly proportional to the toxicity of the SCS (Skovmand *et al*., 2000), thereby affecting comparison between various studies of LC_{50}s (expressed as spores/ml), and not comparisons within the study as is the purpose of this study.

All the anopheline species evaluated in this study were susceptible to *Bti*, with *An. quadriannulatus* appearing to be the most susceptible. Wraight *et al*. (1987) reported that second instar *C. pipens* larvae had an LC_{50} value of 2.3 x 10^3 spores/ml and Lacey and Oldacre (1983) found that second instar *Anopheles quadrimaculatus* and *Anopheles albimanus* had LC_{50} values of 4.6 x 10^4 spores/ml and 5.38 x 10^4 spores/ml respectively. The LC_{50} values obtained in our study are thus within the ranges published for other mosquito species (Panbangred *et al*., 1979; Lacey & Singer, 1982; Wraight *et al*., 1987). Although differences in susceptibility to *Bti* among the *Anopheles* species evaluated was observed, there were not large (log) differences in the LC_{50}s of the five anopheline species evaluated.

Different feeding patterns, such as feeding rates and feeding behaviour, may affect the susceptibility of the anopheline species to *Bti* (Lacey & Singer, 1982; Lee & Lee, 2004). The high susceptibility of *An. quadriannulatus* to *Bti* in our study may be attributed to slightly different feeding behaviour compared to the other anopheline species examined in this study. Future studies may want to study the effects that intra-anopheline differences in behaviour could have on the susceptibility to *Bti*.

The difference in the susceptibility order observed between the LC_{50}s and LC_{90}s can be explained by examining the slopes obtained for the concentration-mortality regression lines. The shallower the slope, the larger

the difference between the LC_{50} and LC_{90} values for the species. The low slope of the concentration-mortality regression obtained for *An. funestus* means that the higher (but not statistically different) LC_{50} of this species is accentuated at the extremes of the regressions, such that significant differences are obtained at the LC_{90}.

The concentration-mortality regression slopes indicate the variability in response to the toxin within the vector population being examined (Jyoti & Brewer, 1999). The regression slopes of *An. quadriannulatus* and *An. merus* were significantly steeper than all the other species examined, thereby indicating that *An. quadriannulatus* and *An. merus* had lower population response variability compared to the other mosquito populations evaluated. The shallower slopes observed for *An. arabiensis* and *An. funestus* suggests that these vector populations are more heterogeneous in their susceptibility to *Bti*, with more individuals in these populations being less susceptible to the *Bti* toxin and hence more of the toxin is required to obtain 90% mortality of these mosquito larvae.

Due to differences in factors such as larval rearing conditions, the different strains of *Bti* examined, and different *Bti* culturing methods, ranking of the susceptibility of species on the basis of LC_{50}s obtained in different studies is not advisable (Skovmand *et al*., 1998). To our knowledge, this study is the first to determine, under the same experimental conditions and using the same *Bti* preparation and strain, the larvicidal activity of *Bti* against the most important malaria vectors (*An. arabiensis*, *An. gambiae*, and *An. funestus*). Although the LC_{50} values did not differ significantly between the anopheline species evaluated, difference in species response variability meant that there were significant LC_{90} differences between the anopheline species. Since LC_{90} values are more relevant for control program application rates, the differences observed in LC_{90} values between the anopheline species have to be taken into consideration in vector control programs. Determination of *Bti* application

rates should consider both the species and the response variability of the populations being targeted, since field populations of the vectors may respond differently to the mosquitoes reared in the laboratory. This study also provided useful information for vector control programs as it shows which of the vectors are most susceptible to *Bti* and provides guidance on the *Bti* concentrations required for effective vector control.

Chapter 3

The larvicidal activity of a genetically engineered cyanobacterium, *Anabaena* PCC 7120#11, against five African *Anopheles* (Diptera: Culicidae) species

3.1 Abstract

The larvicidal activity of a genetically engineered cyanobacterium, *Anabaena* PCC 7120#11, was evaluated against five African *Anopheles* species in laboratory bioassays. The species within the genus *Anopheles* play a major role in the transmission of malaria in Africa and alternative control methods need to be developed to overcome some of the problems arising from current control programs. *Anabaena* PCC 7120#11 (PCC 7120#11), genetically engineered to carry *Bacillus thuringiensis* subspecies *israelensis* (*Bti*) *cry 4Aa*, *cry 11Aa*, and *p20* genes, exhibited larvicidal activity against four of the five anopheline species evaluated (*Anopheles arabiensis, Anopheles merus*, *Anopheles quadriannulatus* species A, and *Anopheles gambiae*). PCC 7120#11 exhibited high larvicidal activity against *An. merus* with an LC_{50} value of 3.90 x 10^5 cells/ml, whereas lower larvicidal activity was exhibited against *An. quadriannulatus* with an LC_{50} value of 15.7 x 10^5 cells/ml. Fifty percent mortality was not obtained even at concentrations as high as 3.20 x 10^7 cells/ml for *An. funestus*, suggesting PCC 7120#11 was not larvicidal to *An. funestus* at ecomonically feasible concentrations. The low larvicidal activity of PCC 7120#11 to *An. funestus* could be due to the absence of the other *Bti* Cry and Cyt proteins in PCC 7120#11 or differences in the structure and/or density of specific midgut toxin receptors for the Cry 4Aa or Cry 11Aa proteins. This study showed that PCC 7120#11 can be used as a potential larvicidal agent against the important malaria vectors such as *An. arabiensis* and *An. gambiae*, especially if used in conjunction with current integrated vector control programs.

3.2 Introduction

Malaria is one of the leading causes of death in Africa, with over a million deaths reported each year (Murray *et al*., 2012). Species within the genus *Anopheles* play a major role in the transmission of malaria in Africa, in particular mosquitoes from the *Anopheles gambiae* Giles complex and the *Anopheles funestus* Giles group (Gillies & De Meillon, 1968).

The *An. gambiae* complex comprises of seven recognized species, and includes the two most important and efficient vectors, *An. gambiae* Giles *sensu stricto* and *Anopheles arabiensis* Patton (Gillies & Coetzee, 1987; Coetzee *et al*., 2000). Within the complex are the minor vectors, *Anopheles bwambae* White, *Anopheles merus* Dönitz, and *Anopheles melas* Theobald, and the non-vector species *Anopheles quadriannulatus* Theobald (species A and B) (Gillies & Coetzee, 1987; Hunt *et al*., 1998; Coetzee *et al*., 2000). Members of the *An. gambiae* complex have been reported from most countries in Africa, with the different species colonizing different breeding habitats (Coetzee *et al*., 2000; Walker & Lynch, 2007).

The *An. funestus* group consists of nine species (Gillies & De Meillon, 1968), with the most important vector of malaria parasites in this group being *Anopheles funestus* Giles *s.s*. (Breman, 2001). *An. funestus* has a wide distribution throughout Africa and is responsible for contributing to one of most recent malaria epidemics in South Africa (Hargreaves *et al*., 2000; Coetzee, 2006).

Vector control is an important part of preventing the spread of malaria, with both the larval and adult stages of the mosquitoes being targeted in integrated vector control programs (Walker & Lynch, 2007; Beier *et al*., 2008). A range of chemical insecticides, such as petroleum oils, organophosphates, organochlorines, pyrethroids, and carbamates have been used successfully in adult and larval vector control programs (Gratz & Pal, 1988; Coosemans &

Carnevale, 1995; Phillips, 2001; Shiff, 2002; Ashley *et al*., 2006; Walker & Lynch, 2007; Okoye *et al*., 2008; Vezenegho *et al*., 2009). However, most larval and adult control programs are hampered by the development of resistance by the vectors, in particular *An. funestus, An. gambiae*, and *An. arabiensis*, to chemical insecticides like dichlorodiphenyltrichloroethane (DDT), dieldrin, carbamates, and pyrethroids (Hargreaves *et al*., 2000; Hargreaves *et al*., 2003; Coetzee, 2004; Coetzee & Fontenille, 2004; Abdalla *et al*., 2008; Okoye *et al*., 2008; Awolola *et al*., 2009; Mouatcho *et al*., 2009; Vezenegho *et al*., 2009). The detrimental effects on the environment and non-target organisms, caused by the chemical insecticides, have prompted the development of more environmentally friendly control agents and/or control programs (Gratz & Pal, 1988; Rose, 2001; Federici *et al*., 2003).

Biological control programs using microbial agents have proven to be effective against several mosquito species, and have the added advantages of being target specific and less harmful to the environment (Becker & Rettich, 1994; Das & Dominic Amalraj, 1997; Lacey *et al*., 2001; Fillinger *et al*., 2003; Pérez-Pacheco *et al*., 2005; Scholte *et al*., 2005). *Bacillus thuringiensis* subspecies *israelensis* (*Bti*) is a Gram-positive, aerobic, spore-forming, bacterium that produces crystalline inclusions that contain crystal (Cry) and cytolytic (Cyt) proteins that are highly toxic to mosquito larvae (Goldberg & Margalit, 1977; Margalit & Dean, 1985; Höfte & Whiteley, 1989; Crickmore *et al*., 1998). Although there is low risk of resistance being developed to *Bti*, due to the specific conditions during which it is activated (Wirth *et al*., 1997; Takken & Knols, 2008), there are several disadvantages to using *Bti* as a control agent. These include its low persistence in the field due to; the adsorption of *Bti* to particles in the water body, inactivation of *Bti* by UV, ingestion of *Bti* by other aquatic organisms, the lack of continued *Bti* synthesis in aquatic environments, and the settling of *Bti* from the mosquito larval feeding zone (Griego & Spence, 1978; Ignoffo *et al*., 1981; Priest, 1992; Ramoska, 1982; Ohana *et al*., 1987; Mulla *et al*., 1990; Blaustein & Margalit, 1991; Becker *et*

al., 1992; Sheeran & Fisher, 1992; Boussiba *et al*., 2000; Manasherob *et al*., 2002).

One strategy to overcome some of the disadvantages of *Bti*, is to clone the *cry* genes of *Bti* into microorganisms inhabiting the breeding sites of mosquitoes and are used by mosquitoes as a food source (Suzuki & Giovannoni, 1996; Armengol *et a*l., 2005; Zheng *et al*., 2007). *Bti cry* genes have been transferred into several aquatic microorganisms such as *Caulobacter crescentus*, *Ancylobacter aquaticus*, *Asticcacaulis excentricus*, and *Enterobacter amnigus*, however moderate toxicity and low mortality to mosquito larvae was reported (Yap *et al*., 1994a; Yap *et al*., 1994b; Liu *et al*., 1996; Tanapongpipat *et al*., 2003; Armengol *et al*., 2005; Zheng *et al*., 2007). Wu *et al*. (1997) inserted the *Bti cry4Aa, cry11Aa*, and *p20* genes under the control of two tandem promoters (cyanobacterial constitutive promoter, $P_{psbA,}$ and *Esherichia coli* T7 early promoter, P_{A1}) into a filamentous nitrogen-fixing cyanobacterium, *Anabaena* sp. strain PCC 7120. The resultant strain (*Anabaena* PCC 7120#11) is able to multiply in the habitats and breeding sites of mosquitoes, maintain its position in the water column due to the presence of gas vacuoles, and is resistant to UV damage (Boussiba *et al*., 2000; Manasherob *et al*., 2002; Manasherob *et al*., 2003). Furthermore, the *Bti* genes had integrated into the chromosome of PCC 7120, resulting in a stable recombinant strain (Lluisma *et al*., 2001).

Although research has shown that *Anabaena* PCC 7120#11, in laboratory bioassays, was a very effective larvicidal agent against *Aedes aegypti* (Wu *et al*., 1997; Lluisma *et al*., 2001; Manasherob *et al*., 2003), to our knowledge no studies, have examined the activity of the recombinant strain *Anabaena* PCC 7120#11 (hereafter referred to as PCC 7120#11) against the larvae of important African anopheline species. The aim of this study was, thus, to evaluate the larvicidal activity of PCC 7120#11 against five African *Anopheles*

species in order to determine if PCC 7120#11 may be used an alternative biological control agent in integrated vector control programs.

3.3 Methodology

3.3.1 Mosquito species

The anopheline mosquito species examined were (origin and colony name provided): *An. arabiensis* (Kanyemba, Zimbabwe; KGB), *An. funestus* (Maputo, Mozambique; FUMOZ), *An. merus* (KwaZulu-Natal, South Africa; MAF), *An. quadriannulatus* species A (Sangwe, Zimbabwe; SANGWE), and *An. gambiae* (Ibadan, Nigeria; NAG). The larvae were obtained from colonies maintained at the National Institute of Communicable Diseases in Johannesburg, South Africa. The larvae were reared in a controlled environment of 25 °C, relative humidity ranging from 60-80%, and a L:D 12:12 hour photocycle with a 45 minute dawn and dusk light regime. *Aedes aegypti* was included as a control in the bioassays. Since the activity of PCC 7120#11 against *A. aegypti* had been previously evaluated (Wu *et al*., 1997), including it as a control would enable us to evaluate if PCC 7120#11 was as larvicidal as had been previously reported. *A. aegypti* was obtained from colonies maintained at the South African Bureau of Standards in Pretoria, South Africa.

3.3.2 Culturing of the cyanobacteria

The wild type *Anabaena* sp. (hereafter referred to as PCC 7120) and recombinant *Anabaena* sp. (PCC 7120#11) were cultured in BG-11 medium (Wu *et al*., 1997), at 30 °C under continuous illumination (2000 lux) with constant agitation on a rotary shaker at 140 revolutions per minute (Lluisma *et al*., 2001; Manasherob *et al*., 2003), until the mid-exponential growth phase.

3.3.3 Harvesting of the cyanobacteria

PCC 7120 and PCC 7120#11 cells were harvested by differential centrifugation at 10 000 *g* (Beckman J2-21 with JA-14 rotor, Beckman Coulter, U.K.) for 25 minutes at 15 °C. The pellet was washed twice and then

resuspended in a minimal volume of sterile distilled water. The cell concentration was determined by using a Neubauer haemocytometer (Superior Co., Berlin, Germany).

3.3.4 Determination of larvicidal activity

For the laboratory bioassays, 98 ml of sterile distilled water was placed into 130 ml plastic cups. Twenty third instar mosquito larvae were selected, rinsed in sterile distilled water, and added to the plastic cups. In the case of *An. merus*, a sterile 5 M saline solution was used instead of sterile distilled water. Two millilitres of the appropriate dilution of either PCC 7120 or PCC 7120#11 was added to the cups. Sterile distilled water was used as the diluent for PCC 7120 and PCC 7120#11. The larvicidal activity was determined after 24 hours incubation in a controlled environment of 25 °C, relative humidity of 60%, and a L:D 12:12 hour photocycle. Larvae were presumed dead if they did not move when prodded. An untreated control (2 ml of sterile distilled water) was included in the bioassays. If mortality in the controls (PCC 7120 and untreated) exceeded 5%, the test was discarded and repeated. Each bioassay was repeated in triplicate on different days. The lethal concentration (LC_{50} and LC_{90}) of each species was determined by probit analysis (Finney, 1971). For each species, probit analysis was based on the mortality data obtained from five PCC 7120#11 concentrations.

3.4 Results

The concentration-mortality data for the mosquito species examined are summarized in Table 3.1. The heterogeneity factors for the different mosquito species evaluated were all less than one indicating a good fit of the concentration-mortality data to the probit model (Champ & Campbell-Brown, 1970).

The LC_{50} values differed significantly between the mosquito species examined (Table 3.1). PCC 7120#11 was not larvicidal to *An. funestus*, even

at concentrations as high 3.2 x 10^7 cells/ml (Table 3.1). The LC_{50} value for *A. aegypti* was significantly lower than that of the anopheline species evaluated, indicating that *A. aegypti* was very susceptible to PCC 7120#11. Among the anopheline species, PCC 7120#11 was most effective against *An. merus* larvae (3.9 x 10^5 cells/ml) (Table 3.1). The larvae of *An. quadriannulatus* were 11 times less susceptible (based on LC_{50} ratios) to PCC 7120#11 than *A. aegypti* larvae, whereas *An. merus* larvae were 4 times more susceptible than *An. quadriannulatus* larvae. The LC_{90} values of *An. merus* and *A. aegypti* were significantly lower than the LC_{90} values of *An. arabiensis*, *An. quadriannulatus*, and *An. gambiae* (Table 3.1). The LC_{90} of *An. arabiensis* was significantly lower than the LC_{90} of *An. quadriannulatus* or *An. gambiae* (Table 3.1).

Table 3.1 Probit analysis of concentration-mortality data for *Anabaena* PCC 7120 #11 against third instar mosquito larvae.

Species	LC_{50} (10^5 cells/ml) [1]	LC_{90} (10^5 cells/ml) [1]	Slope ± SE [2]	Heterogeneity [3]
A. aegypti	1.42 (1.12-1.76)[a]	8.21 (6.05-12.3)[a]	1.70±0.15[a]	0.75
An. merus	3.90 (3.58-4.17)[b]	9.30 (8.0-11.4)[a]	3.37±0.31[b]	0.62
An. arabiensis	8.10 (7.62-8.56)[c]	14.3 (12.8-16.5)[b]	5.18±0.46[c]	0.19
An. gambiae	12.3 (11.4-13.3)[d]	35.1 (29.5-44.9)[c]	2.81±0.24[d]	0.49
An. quadriannulatus	15.7 (14.3-17.3)[e]	43.0 (35.9-54.9)[c]	2.93±0.26[bd]	0.92
An. funestus	ND[4]	ND	ND	ND

[1] Values in brackets show the 95% fiducial limits (FLs). Values in a column followed by the same letters are not significantly different (non-overlapping 95% FLs).

[2] Slope ± standard error. Values followed by the same letters are not significantly different ($p > 0.05$).

[3] Heterogeneity factor = χ^2 / d.f. (degrees of freedom).

[4] Not determined. Fifty percent mortality was not obtained even at concentrations as high as 3.2 x 10^7 cells/ml.

3.5 Discussion

Concentration-mortality regression slopes indicate the variability in response to a toxin within the vector population being examined (Jyoti & Brewer, 1999). In this study, *An. arabiensis* had a significantly steeper slope than the other species evaluated, thereby suggesting that *An. arabiensis* had lower

population response variability or reduced heterogeneity in their populations compared to the other mosquito species examined. In contrast, larvae in the *A. aegypti* population were more heterogeneous in their response to PCC 7120#11 than the other anopheline species evaluated, as indicated by their statistically shallowest concentration-mortality regression slope. The statistically shallower slopes of the concentration-mortality regression lines observed for *An. gambiae* and *An. quadriannulatus* indicate that there are larger differences between the LC_{50} and LC_{90} values for these species than for the other species evaluated.

Previous studies, using PCC 7120#11, reported that third instar *A. aegypti* larvae had an LC_{50} of 0.9 x 10^5 cells/ml (Wu *et al*., 1997), which is comparable to the LC_{50} of 1.4 x 10^5 cells/ml obtained for *A. aegypti* in this study. The laboratory bioassays confirmed that the efficiency of PCC 7120#11 was similar to that previously reported by Wu *et al*. (1997), indicating that PCC 7120#11 larvicidal activity in our laboratory was within the range of previous studies. The slight variation observed in LC_{50}s between our study and the study of Wu *et al*. (1997) may be due to differences in experimental conditions such as the water quality or containers used in the bioassays, different PCC 7120#11 culturing conditions, differences in rearing conditions of the larvae, and natural variations in the larval populations (Otieno-Ayaya *et al*., 2008).

In this study, the *A. aegypti* larvae were more susceptible to PCC 7120#11 than the anopheline species evaluated. It has previously been reported that *A. aegypti* larvae are more susceptible to the *Bti* toxins than anopheline species (Tyrell *et al*., 1979; Lacey & Singer, 1982; Majori *et al*., 1987; Wraight *et al*., 1987; Dominic Amalraj *et al*., 2000; Lee & Lee, 2004). The differences observed in susceptibility between the *Anopheles* and *Aedes* larval species could be a result of different feeding behaviours and feeding rates (Lacey & Singer, 1982; Merritt *et al*., 1992; Lee & Lee, 2004). The *Aedes* species are

bottom feeders and can thus filter-feed throughout the water column, whereas the anopheline species are air-water interface feeders (Merritt *et al*., 1992). This difference in feeding behaviour may result in higher larvicidal activity of *A. aegypti*, as the *A. aegypti* larvae may ingest more PCC 7120#11 by feeding on any settled PCC 7120#11.

The larvicidal activity of *Bti* Cry toxins is often correlated to the high affinity binding of the toxin to the specific membrane-bound receptors in the larval midgut (Hofmann *et al*., 1988; Van Rie *et al*., 1990; Ravoahangimalala *et al*., 1993; Ravoahangimalala & Charles, 1995), thus the differences observed in susceptibility to PCC 7120#11 within the anopheline species and between the anopheline species and the *A. aegypti* larvae could be due to differences in the structure and/or density of specific midgut toxin receptors for Cry4Aa or Cry11Aa proteins. This suggests that there may be inherent physiological differences in the structure and density of the larval midgut toxin receptors of the anopheline species evaluated, in particular between *An. funestus* in the *An. funestus* group and members of the *An. gambiae* complex, that could affect the variability in larvicidal activity (De Maagd *et al*., 2001; Otieno-Ayaya *et al*., 2008). However, this hypothesis would need to be confirmed by detailed studies of the binding of the different Cry proteins to the midgut receptors of the different species evaluated.

Another factor to consider in interpreting the observed differences in larvicidal activity between the susceptible anopheline species and *An. funestus* is the combination of the toxins present in the recombinant clone. *Bti*, which naturally produces crystals that contain the toxins Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa and Cyt2Ba (Höfte & Whiteley, 1989; Schnepf *et al*., 1998; Ben-Dov *et al*., 1999; Berry *et al*., 2002), has higher larvicidal activity against mosquitoes than recombinant clones containing a subset of toxins (Delécluse *et al*., 1993; Poncet *et al*., 1995; Schnepf *et al*., 1998). For example, Delécluse *et al.* (1993) showed that Cry4Aa was toxic to fourth instar *A.*

aegypti, *Culex pipiens*, and third instar *Anopheles stephensi* larvae, whereas Cry4Ba was only toxic towards *A. aegypti* and *An. stephensi* larvae. Furthermore, the larvicidal activity of a combination of both Cry4Aa and Cry4Ba was higher than that of the individual toxins, but not as high as the *Bti* crystal (Delécluse *et al.*, 1993). In a previous study (Ketseoglou *et al*., 2011), *Bti* strain HD522 was evaluated against the same anopheline species, with *An. funestus* larvae having an LC_{50} (28.5 ng/ml) that was statistically similar to the LC_{50} values obtained for *An. arabiensis*, *An. gambiae*, and *An. merus* larvae. The low larvicidal activity of PCC 7120#11 to *An. funestus* could be due to the absence of the other Cry and Cyt proteins in PCC 7120#11, which produces Cry4Aa and Cry11Aa proteins. Further research is required in order to determine which combinations of the Cry and Cyt proteins would result in high larvicidal activity against *An. funestus* larvae.

This study showed the larvicidal activity of a *Bti* Cry-protein producing recombinant *Anabaena* (PCC 7120#11) against five African anopheline species. With the exception of *An. funestus*, PCC 7120#11 was shown to be larvicidal to all the *Anopheles* species evaluated at economically practical concentrations. Although PCC 7120#11 was not highly larvicidal to *An. funestus* at economically practical concentrations, it has potential to be used as a larvicidal agent in areas or regions where *An. funestus* is not the predominant vector. Alternatively, PCC 7120#11 can be applied as a larvicidal agent as part of integrated vector control programs that target adult *An. funestus* mosquitoes and *An. arabiensis* larvae.

Chapter 4

The use of Standardized Aquatic Microcosms in evaluating the effect of *Bacillus thuringiensis* subspecies *israelensis* on target and non-target organisms

4.1 Abstract

Malaria, one of the most deadly vector-borne diseases in the world, is transmitted by the bite of some species of female *Anopheles* mosquitoes. The malaria vector of interest in this study is *Anopheles arabiensis*, the most widespread vector of malaria in southern Africa. *Bacillus thuringiensis* subspecies *israelensis* (*Bti*) has been proven to be highly insecticidal to larvae of mosquitoes and blackflies, however few studies have comprehensively evaluated the effect of *Bti* on non-target organisms. The objective of this study was to evaluate the effects of *Bti* on several invertebrates and other variables in a synthetic multi-species system, the Standardized Aquatic Microcosm (SAM). The SAM is initiated in a chemically defined medium with synthetic sediment and uses nine different species of photosynthetic microorganisms (PMOs) including algae and cyanobacteria, and invertebrates. The larvicidal activity of *Bti* did not persist for longer than 7 days. The data showed that certain environmental conditions like abundance of PMOs or the presence of potential invertebrate competitors could reduce the larvicidal activity of *Bti*. The nitrates, nitrites, and phosphates were utilized by day 25 in the control and *Bti* microcosms. Increases in PMOs were coupled to a decrease in the inorganic nutrients. Decreases in PMOs were observed as the invertebrate populations, in particular *Daphnia pulex* populations, increased. *Coleps* sp. populations peaked before the other invertebrates. The general trends between the control and *Bti* microcosms were similar, with no statistically significant differences being recorded when the main effect of treatment was evaluated. Although the interaction between time and treatment was significantly different on day 56 in the *D. pulex* populations, the interaction between time and treatment on the other variables evaluated was not significant. This study showed that, at the concentration applied to the microcosms, *Bti* did not have any adverse effects on the invertebrates or other variables evaluated. *Bti* is thus an environmentally friendly larvicidal agent.

4.2 Introduction

Malaria is one of the most important vector-borne diseases in the world, with an annual worldwide incidence leading to over 1.2 million deaths (Murray *et al*., 2012). Malaria generally affects people living in tropical and subtropical regions (Collins & Besansky, 2004). It is transmitted by the bite of female *Anopheles* mosquitoes (Cowman & Crabb, 2006). There are roughly 400 species of *Anopheles*, of which only 30 species are important vectors of malaria (Bruce-Chwatt, 1985). The most efficient malaria vectors are *Anopheles gambiae* Giles *sensu stricto*, *Anopheles arabiensis* Patton, and *Anopheles funestus* Giles (Gillies & Coetzee, 1987; Breman, 2001). The malaria vector of interest in this study is *An. arabiensis*, which is the most widespread vector of malaria in southern Africa. *An. arabiensis* is one of the seven recognized species belonging to the *An. gambiae* complex (Coetzee *et al*., 2000; Moreno *et al*., 2007). The females exhibit variable behaviour being both anthropophilic and zoophilic, as well as endophilic and exophilic (Gillies & Coetzee, 1987).

Current vector control programs include indoor residual house spraying, insecticide-treated fabrics like bed nets, and larvicidals (Coosemans & Carnevale, 1995; Hougard *et al*., 2002; Ashley *et al*., 2006; Coetzee, 2006; Walker & Lynch, 2007; Okoye *et al*., 2008). However, some of the major problems with current control programs include chemical insecticide resistance, and detrimental effects on humans and the environment by the chemical insecticides (Ranson *et al*., 2001; Rose, 2001; Hougard *et al*., 2002; Shiff, 2002; Federici *et al*., 2003; Coetzee, 2006). *An. arabiensis* has been shown to be resistant to several important chemical insecticides such as dichlorodiphenyltrichloroethane (DDT), pyrethroids, and organophosphates (Hargreaves *et al*., 2000; Hargreaves *et al*., 2003; Mouatcho *et al*., 2009). The continual problem of malaria-carrying vectors exhibiting resistance to chemical insecticides, and the harmful effects of the chemical insecticides on the environment and non-target organisms has lead to an increase in the

demand for insecticides of biological origin (biopesticides) that are environmentally friendly, more target specific, and more economical (Copping & Menn, 2000; Rey *et al*., 2001; Hynes & Boyetchko, 2006).

Biopesticides are usually derived from natural materials such as microbial organisms, entomophagous nematodes, secondary metabolites from microorganisms, and plant-derived pesticides (Copping & Menn, 2000). Currently, there are several beneficial microorganisms used in the agricultural industry with *Bacillus thuringiensis* Berliner (*Bt*) based insect-control products being the most successful (Armengol *et al*., 2005). *Bt* is an aerobic, spore-forming, Gram-positive bacterium that produces parasporal crystals containing one or more insecticidal crystal proteins (ICPs) (Schnepf *et al*., 1998; Kaur, 2000; Crickmore, 2006). The crystal (Cry) proteins are solubilised upon ingestion and bind to specific sites on the microvilli brush-border membranes in the midgut of the insect, causing pores to form which ultimately leads to the death of the insect (Tojo & Aizawa, 1983; Thomas & Ellar, 1983; Höfte & Whiteley, 1989; Ravoahangimalala & Charles, 1995; Schnepf *et al*., 1998). The specific toxicity of Cry proteins against target insects is one of the reasons for the use of *Bt* as a biopesticide (Kaur, 2000; Crickmore, 2006).

Bacillus thuringiensis subspecies *israelensis* (*Bti*) has been proven to be highly insecticidal to larvae of mosquitoes and blackflies (Goldberg & Margalit, 1977; Tyrell *et al*., 1979; Van Essen & Hembree, 1980; Ignoffo *et al*., 1981; Gunasekaran *et al*., 2002). The success of *Bti* in the laboratory lead to the development and use of commercial mosquitocides such as VectoBac®, Teknar®, Batimos®, and Arobe® (Becker & Rettich, 1994; Federici *et al*., 2003; Gunasekaran *et al*., 2004). Several studies examined the larvicidal activity of the commercial formulations against several mosquito species, in both the laboratory and the field (Majori & Ali, 1984; Lacey & Inman, 1985; Majori *et al*., 1987; Karch *et al*., 1991; Becker & Rettich, 1994; Skovmand & Sanogo,

1999; Dominic Amalraj *et al*., 2000; Fillinger *et al*., 2003; Gunasekaran *et al*., 2004; Zahiri *et al*., 2004; De Araújo *et al*., 2007).

Studies have also evaluated the effect of *Bti* on several key non-target organisms (Colbo & Undeen, 1980; Ali, 1981; Mulla *et al*., 1982; Gunasekaran *et al*., 2004), however no studies have comprehensively evaluated the effects on *Bti* in a standardized multi-species microcosm. Evaluating *Bti* in a multi-species microcosm test is very valuable since it allows the assessment of *Bti* on several species exposed simultaneously under the same conditions (Clément & Cadier, 1998). Environmental risk assessment of pesticides is usually based on toxicity studies carried out on single compounds in single-species tests (an alga, or *Daphnia magna*) (Cuppen *et al*., 2002; Duchet *et al*., 2008). However, single-species tests ignore the importance of interactions among species (Kimball & Levin, 1985; Swartzmann *et al*., 1990; Traunspurger *et al*., 1996; Crane, 1997). Furthermore, single-species tests cannot be used to predict the behaviour of large-scale systems (Kimball & Levin, 1985; Crane, 1997). Laboratory multi-species microcosm tests can simulate field conditions, can be standardized, and can be reproduced (Taub *et al*., 1988; Conquest & Taub, 1989).

The microcosm protocol to be evaluated in this study was based on the Standardized Aquatic Microcosm (SAM) protocol (Taub, 1989). The term “standardized” refers to microcosms being initiated with the same chemically defined medium and most, if not all, the organisms are known and added at known concentrations (Taub *et al*., 1988; Taub, 1989; Taub, 1997), thereby reducing variability. Standardizing microcosm studies enables comparisons of data from diverse microcosms and allows for the replication of results (Krimsky *et al*., 1995; Taub *et al*., 1988; Taub, 1989; Taub, 1997). The SAM experiments are complex enough to demonstrate interactions within and between trophic levels, but are not as complex as most natural communities wherein it is usually possible to track only the few most abundant species

(Taub, 1992; Taub, 1997). This allows for more focussed analysis on the ecological relationships. The rapidity with which the SAM experiments can be conducted and analyzed is another major advantage (Taub, 1992; Taub, 1997).

The objective of this study was to evaluate the effect of *Bti* on target and non-target organisms in a standardized multi-species microcosm using the SAM protocol (Taub, 1989). This study is the first, to our knowledge, that utilized a standardized, reproducible microcosm to evaluate the effects of *Bti* on non-target organisms and the target malaria vector, *An. arabiensis*. This study provided valuable information on the effect of *Bti* on the different trophic levels, and also determined the effect primary and secondary producers had on the larvicidal activity of *Bti*.

4.3 Methodology

4.3.1 Microcosms

The microcosms were set up according to the SAM protocol as described in the ASTM E1366-02 Standard Practice for Standardized Aquatic Microcosms: Fresh Water (ASTM, 2002) with a few modifications. The microcosm set up will be described briefly. The temperature in a controlled room was set at 21 ± 2 $^{\circ}$C. Cool white lights were used for illuminance (4700 lux) with a L:D 12:12 hour photoperiod.

The microcosms consisted of 4 litres (l) sterile glass jars containing 3 l of autoclaved chemically defined medium (T82MV) (ASTM, 2002). Autoclaved silica sand enriched with chitin and cellulose powder was added to the glass jars. Six jars (replicates) were allocated for *Bti* and six jars (replicates) for the control, with an additional five prepared in case of breakages within the first week (ASTM, 2002). The glass jars were covered individually with square glass plates to minimize contamination and evaporation. Nine different species of photosynthetic microorganisms (PMOs) which includes algal and

cyanobacterial species were added to the jars on day 0 (start of the microcosms) and on day 4, four species of invertebrates were added. After 7 days, quality control was performed to ensure the well-being of the communities in the microcosms (ASTM, 2002). On day 7, a known concentration of *Bti* strain HD-522 was added. Twice a week the organism abundance (PMOs and invertebrates) was measured, in addition the pH, turbidity, total volume, inorganic nutrients, and dissolved oxygen were determined. The larvicidal activity of *Bti* was determined by the weekly addition of 30 third instar *An. arabiensis* larvae. The mortality of the larvae was recorded 24 hours post-inoculation (p.i.), after which they were removed. Larvae were added to the control microcosms without the addition of *Bti*.

4.3.2 Culturing of PMOs

Anabaena cylindrica, *Ankistrodesmus* sp., *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Oscillatoria simplicissima*, *Scenedesmus obliquus*, *Selenastrum capricornutum*, *Stigeoclonium* sp., and *Ulothrix* sp., were purchased from the University of Texas (UT) Algal Culture Collection. The PMOs were cultured under continuous illuminance (2000 lux), at 25 $^{\circ}$C, in individual 1 l Erlenmeyer flasks containing sterile T82MV media (Taub, 1989). The cells were counted using a Palmer cell and were added to the microcosms at a concentration of 5 x 10^3 cells/ml. Filamentous PMOs or PMOs that tended to form clumps were, prior to counting and before being added to the microcosms, vigorously shaken in sterile jars with sterile glass beads (ASTM, 2002). The counts were calculated using the equation presented in the protocol (ASTM, 2002).

4.3.3 Culturing of invertebrates

Daphnia pulex (obtained from colonies at the Department of Water Affairs, Pretoria, South Africa) was cultured in sterile 3 l glass jars containing sterile hard water (123 mg/l $MgSO_4$; 96 mg/l $NaHCO_3$; 4.0 mg/l KCl; 60 mg/l $CaSO_4.2H_2O$). They were fed a mixture of yeast, fish food (TetraMin®), and

alfalfa (Mpho Khumalo, pers. comm.). *D. pulex* were cultured at 18 °C with a L:D 18:6 hour photoperiod. The cultures were fed three times a week and sub-cultured every 2 weeks to avoid over-population. Sixteen *D. pulex* (three adults, three adults with eggs, and 10 small) were added to each of the microcosm jars on day 4 (ASTM, 2002).

The ostracod, *Cyprinotus africanus*, was cultured in sterile glass beakers (500 ml) containing sterile T82MV media (Taub, 1989). Ostracods were fed a mixture of *Stigeoclonium* sp. and *C. vulgaris* cultures. *C. africanus* were cultured at 18 °C with a L:D 18:6 hour photoperiod. On day 4 of the experiment, *C. africanus* were removed from the beakers, rinsed in sterile T82MV media, and then added to each microcosm jar (6 ostracods per jar) (ASTM, 2002).

The protozoans (*Coleps* sp.) and the rotifers (*Lepadella* sp.) were cultured in Petri dishes containing T82MV media, yeast, and *C. vulgaris*. They were cultured at 25 °C under L:D 12:12 hour photoperiods. Three hundred protozoans and 90 rotifers were added to each microcosm jar on day 4 of the experiment (ASTM, 2002).

4.3.4 Culturing of *Bti*

Bti HD-522 was supplied by the Bacillus Genetic Stock Centre (BGSC; Ohio State University, Columbia, Ohio, U.S.A.). HD-522 was streaked onto nutrient agar plates, incubated at 30 °C overnight, and stored at 4 °C. A starter culture of 20 ml of nutrient broth-yeast extract sporulation medium (NYSM) (Myers & Yousten, 1980), in 100 ml Erlenmeyer flasks, was inoculated with *Bti* from the streak plate. The starter culture was incubated at 30 °C for 12 hours on a rotary shaker at 240 revolutions per minute (rpm). A loopful of the starter culture was then seeded into fresh 100 ml NYSM media in a 500 ml Erlenmeyer flask, incubated at 30 °C at 240 rpm until 95% sporulation was obtained. Sporulation was determined by phase contrast microscopy. The

spores and crystals were harvested through differential centrifugation (Beckman model J2-21 with JA-14 rotor, Beckman Coulter, U.K.) at 6000 *g* for 20 minutes. The supernatant was discarded and the pellet was washed twice by differential centrifugation in sterile distilled water. After the final wash the pellet was suspended in a small volume of sterile distilled water and stored at 4 ^{o}C.

4.3.5 Determination of *Bti* concentration

A 10^{-2} dilution of the harvested spore-crystal suspension (SCS) was prepared (in triplicate) with sterile distilled water and the test tubes were heated at 65 ^{o}C for 30 minutes with agitation every 5 minutes. The tubes were then rapidly cooled under running tap water. Duplicate serial dilutions of the SCS (10^{-2} to 10^{-9}) were prepared. After 12 hours incubation at 30 ^{o}C, colonies on the plates were counted using a colony counter. A concentration of 3.5 x 10^{4} spores/ml, resulting in 90% mortality (LC_{90}) of *An. arabiensis* larvae (data not shown), was distributed evenly over the surface of the microcosms (6 jars in total) on day 7. The same concentration of *Bti* was added to the microcosms every alternate week.

4.3.6 Mosquito species

An. arabiensis (origin and colony name provided) (Kanyemba*,* Zimbabwe; KGB), was obtained from colonies maintained at the National Institute of Communicable Diseases in Johannesburg, South Africa. The larvae were reared in a controlled environment of 25 ^{o}C, relative humidity of 60%, and a L:D 12:12 hour photoperiod with a 45 minute dawn and dusk light regime. Thirty third instar mosquito larvae were added to the control and *Bti* microcosm jars on day 7 (after *Bti* had been added to the *Bti* microcosms). Thereafter, larvae were added to the microcosms every week. Mortality was recorded 24 hours p.i., after which all larvae were removed.

4.3.7 Sample collection

Sampling and measurement of organism abundances and other variables were performed twice a week on the same days of the week (Tuesdays and Fridays). The total volume in the microcosms was recorded every sampling day. The turbidity of the microcosms was determined on the sampling day, before any samples were removed or any other readings taken. Twenty millilitres of the unstirred microcosm was removed with a sterile syringe, placed into small, clean glass vial, and the turbidity measured using a calibrated turbidimeter (Turbiquant®1500T, Merck, Darmstadt, Germany). The 20 ml was poured back into the microcosm and the glass vial rinsed with sterile distilled water. One glass vial was set aside for the control microcosms and another glass vial for the microcosms containing *Bti*.

Dissolved oxygen (DO) was measured in the morning (before lights on) and afternoon (before lights off) on the day prior to sampling (Mondays and Thursdays) and again in the morning (before lights on) on the day of sampling (Tuesdays and Fridays) (ASTM, 2002). The net oxygen gain or photosynthesis was determined by subtracting the DO measurement taken in the morning before sampling day from the DO measurement taken in the afternoon before the sampling day (ASTM, 2002). The respiration or net oxygen loss was determined by subtracting the DO measurement taken on the morning of sampling from the DO measurement taken on the afternoon prior to the sampling day (ASTM, 2002). The pH of the microcosms was also determined at the same time periods as the DO measurements were taken. DO and pH was determined using a HI 9828 multiparameter HANNA probe (HANNA Instruments, Rhode Island, U.S.A.).

After DO, pH and turbidity were measured, the microcosms were mixed using a sterile spatula and the sides of the jars were scraped as completely as possible with a sterile rubber spatula (ASTM, 2002). Twenty millilitres was removed and filtered through 0.45 µm 25 mm cellulose acetate filters

(Sartorius Biotech, Goëttingen, Germany) and the filtrate kept for chemical analysis. Another 4 ml was removed with 2 ml being placed into sterile microcentrifuge tubes for PMO counts and 2 ml for *Coleps* sp. and *Lepadella* sp. counts. The PMO counts were performed using a Palmer cell, examining a maximum of 65 fields of view (ASTM, 2002). The rotifers and protozoans were counted in 100 µl aliquots in 96 well micro-titre plates. A total volume of 2 ml was evaluated (ASTM, 2002). The *D. pulex* and *C. africana* populations were counted *in situ* but if they were too numerous to count, then 100 ml of the stirred microcosm was removed and counted in a Petri dish and repeated as required. The removed aliquots were returned to the microcosms after the counting was performed.

4.3.8 Chemical analysis

Twenty millilitres from the mixed microcosms were filtered through 0.45 µm 25 mm cellulose acetate filters (Sartorius Biotech, Goëttingen, Germany) and the filtrates were analyzed for nitrate, nitrite, ammonium, and phosphate concentrations using photometric Spectroquant® test kits (Merck, Darmstadt, Germany). The inorganic concentrations were analyzed photometrically using the photometer Spectroquant® NOVA 60 (Merck, Darmstadt, Germany). All samples were processed on the sampling day.

4.3.9 Statistical analysis

A repeated measure analysis of variance (rm-ANOVA) with a between-subject effect (treatment) and a within-subject effect (time) was used to assess the effects of the treatment on each variable evaluated. When rm-ANOVA indicated a significant effect of treatments, data were analyzed further by *post hoc* Bonferroni tests to detect when differences between treated and control microcosms occurred. Log_{10} transformations were applied to all the counts and the data were analyzed using Statistica 7.1 (Statsoft, Tulsa, OK, U.S.A.). Differences were considered significant at $\alpha = 0.05$ for all tests.

4.4 Results

4.4.1 Target effects

Bti was added to the microcosms at an LC_{90} concentration, determined by preliminary trials (data not shown), on day 7 and then every alternate week (days 21, 35, and 49). Twenty-four hours p.i. of *Bti* (day 8), mortality of the third instar *An. arabiensis* larvae was high at 87 ± 4% (mean values ± SE; n=180; 6 replicates of 30 larvae per replicate) decreasing to 15 ± 5% mortality a week later. The addition of the same concentration of *Bti* on day 21 resulted in a reduced mortality (24 hours p.i.) of 65 ± 15% with no mortality recorded a week later (day 28). Further additions of *Bti* on days 35 and 49 saw a further drop in larval mortality to 27 ± 18% and 39 ± 17% respectively. No *An. arabiensis* larval mortality was recorded a week after *Bti* was added on day 35 or a week after *Bti* was added on day 49. No *An. arabiensis* mortality was recorded in any of the control microcosms.

4.4.2 Effects on variables

The change in nitrate concentration over time was very similar in the control and *Bti* microcosms (Figure 4.1). The main effect of treatment (*Bti* or control) on nitrate was not significant ($F_{1,10}$=0.618; p=0.4500). The interaction between treatment and time was also not significant ($F_{13,130}$=0.855; p=0.6022). For each of the variables (chemical, physical, and invertebrates) evaluated, the main effect of time was, as expected, a significant effect and will subsequently not be mentioned further. Nitrate was reduced from its initial concentration of 8 mg/l to almost undetectable limits by day 25, with continuing low concentrations until day 53 (Figure 4.1). Nitrate concentration depletion started from day 0 with the decline in nitrate concentration corresponding to the increases in the PMO cell concentration (Figure 4.4).

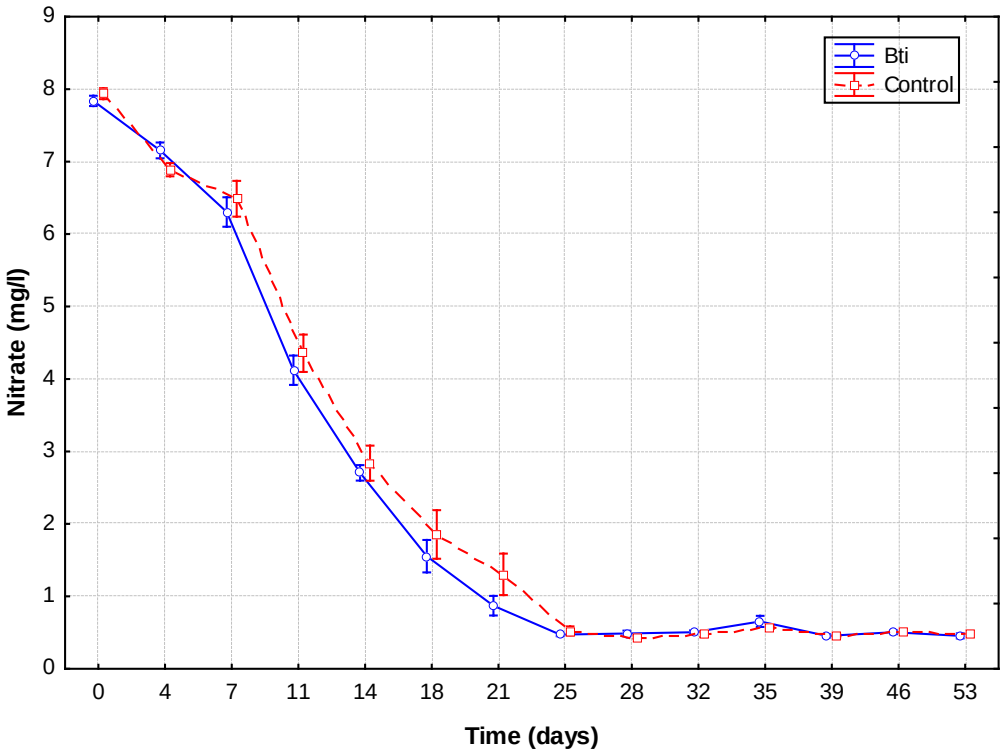

Figure 4.1 Change in nitrate concentration in control and *Bti* microcosms over time. Values shown are mean value ± SE, n=6 for each data point.

For nitrite concentrations, the interaction between time and treatment was not significant ($F_{13,130}$=0.350; p=0.9819) and the main effect of treatment was also not significant ($F_{1,10}$=0.220; p=0.6490) (Figure 4.2). Throughout the experiment, the changes in the nitrite concentrations in the *Bti* microcosms were similar to the changes observed in the control microcosms (Figure 4.2). Initially nitrite concentrations were approximately 0.7 mg/l and by day 25 had decreased to almost undetectable levels. Nitrite concentrations remained low until day 53 (Figure 4.2).

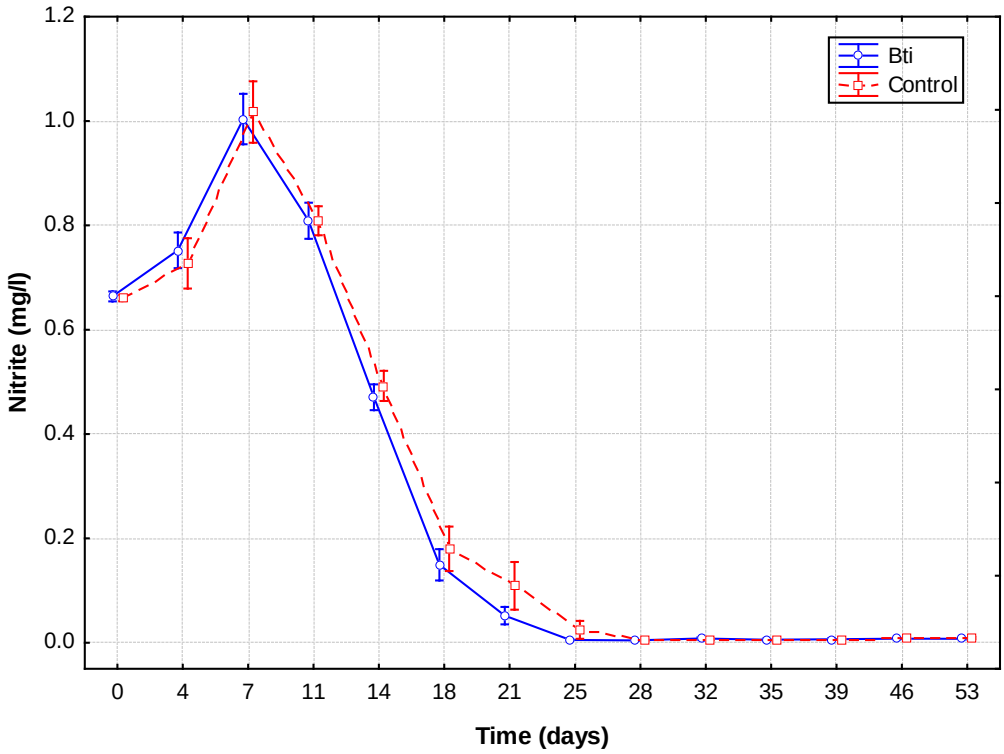

Figure 4.2 Change in nitrite concentration in control and *Bti* microcosms over time. Values shown are mean ± SE, n=6 for each data point.

Initial concentrations of phosphate were approximately 1.3 mg/l, decreasing to just above 0 mg/l by day 25 (Figure 4.3). The main effect of treatment was not significant ($F_{1,10}$=0.2638; p=0.6187), as was the interaction between treatment and time ($F_{13,130}$=0.4183; p=0.9610). Although there was no significant difference between treatments, a slight difference in profiles between treatments was observed after day 25 (Figure 4.3). After day 25 there was an increase in the concentration of phosphate in the control and *Bti* microcosms, with the phosphate concentration in the *Bti* and control microcosms reaching approximately 0.4 mg/l and approximately 0.2 mg/l by day 53, respectively (Figure 4.3). The increase in standard error bars observed after day 25 suggests that there is variation within the microcosms probably due to slight variations in one of the other variables examined like increases in *D. pulex* populations or PMO concentrations (which would have an effect on the rate of phosphate recycling).

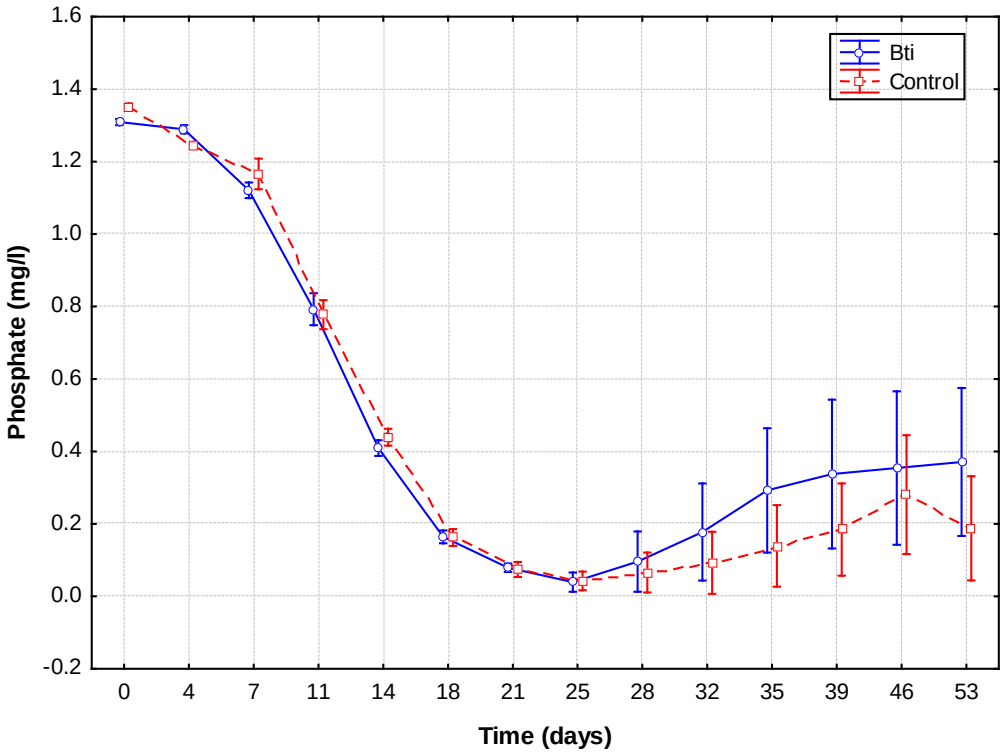

Figure 4.3 Change in phosphate concentration in control and *Bti* microcosms over time. Values shown are mean ± SE, n=6 for each data point.

As can be seen in Figure 4.4, the log transformed total PMO cell concentrations increased rapidly between day 0 and day 4 followed by a gradual increase until day 25 (log PMO cell concentration of 6.5 cells/ml). From day 25 until day 56, there was a gradual decrease in total PMO cell concentration. The changes in log PMO cell concentrations over time were similar in the control and *Bti* microcosms (Figure 4.4). The interaction between time and treatment was not significant ($F_{16,160}$=0.419; p=0.9760). The main effect of treatment on log PMO cell concentration was also not significant ($F_{1,10}$=0.280; p=0.6084). After day 25, the total log PMO cell concentration decreased gradually until the end of the experiment. The concentration of the individual PMO species varied during the experiment (data not shown). The numerically dominant PMO species present in the microcosms by day 25, were *Ankistrodesmus* sp. and *A. cylindrica* (mean log cell concentration of 6.1 cells/ml and 5.9 cells/ml, respectively), and the less abundant PMO species were the smaller, unicellular species like *S. obliquus* (mean log cell concentration of 4.9 cells/ml), *C. vulgaris* (mean log cell concentration of 4.9

cells/ml), *C. reinhardtii* (mean log cell concentration of 4.9 cells/ml), and *S. capricornutum* (mean log cell concentration of 4.5 cells/ml). Towards the end of the 56 day period, the dominant PMO species were still *A. cylindrica* and *Ankistrodesmus* sp. (data not shown). The increase in standard error bars after day 25 (Figure 4.4) could be due to slight replication differences in the *D. pulex* populations, leading to variations in the total PMOs consumed.

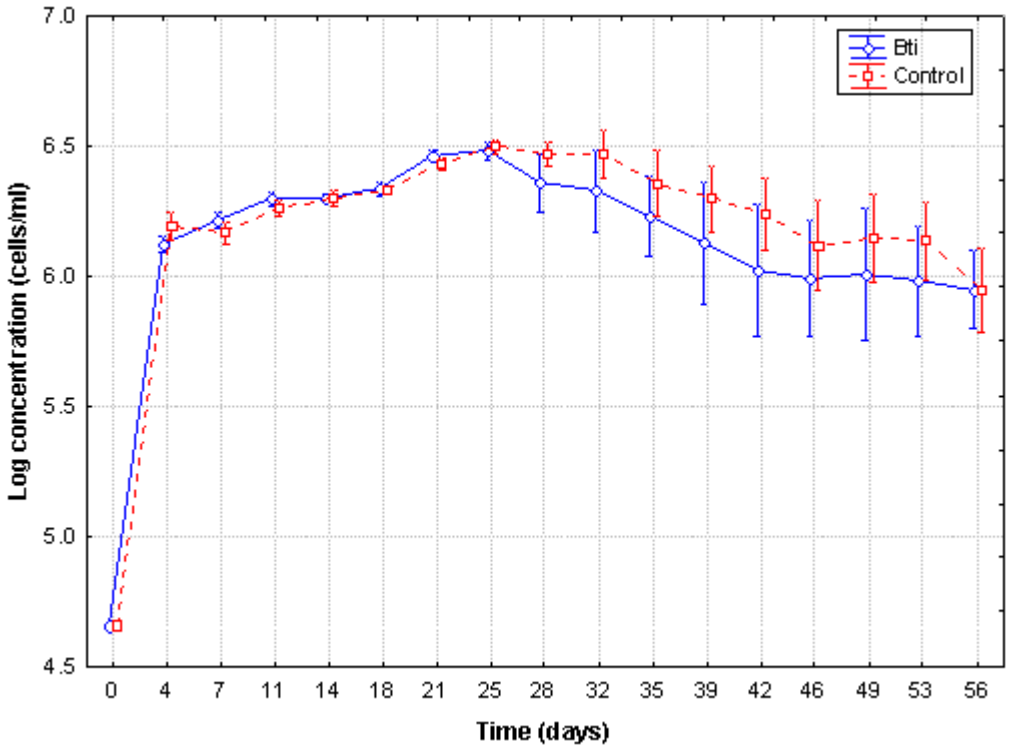

Figure 4.4 Change in concentration of total photosynthetic microorganisms in control and *Bti* microcosms during the 56-day experiment. Values shown are mean ± SE, n=6 for each data point.

Figure 4.5 shows the change in the concentration of *D. pulex* populations throughout the 56 day period. The main effect of treatment was not significant ($F_{1,10}$=0.0002; p=0.9888). Although the interaction between time and treatment was significant ($F_{15,150}$=0.3622; p<0.005), the *post hoc* Bonferroni test showed that the significant difference only occurred at day 56 (at the end of the experiment). The *D. pulex* populations exhibited an initial, slow increase (Figure 4.5). The *Bti* microcosms had slightly higher *D. pulex* concentrations than the control microcosms (not a significant difference as shown by the rm-

ANOVA and *post hoc* test) reaching a peak at day 32 and day 35 respectively, coinciding approximately with a decrease in PMO concentration (Figure 4.4, day 28). After day 32, the concentration of *D. pulex* populations in the *Bti* microcosms gradually declined. From day 46 there was a rapid increase in the concentration of the *D. pulex* populations in the control microcosms (Figure 4.5).

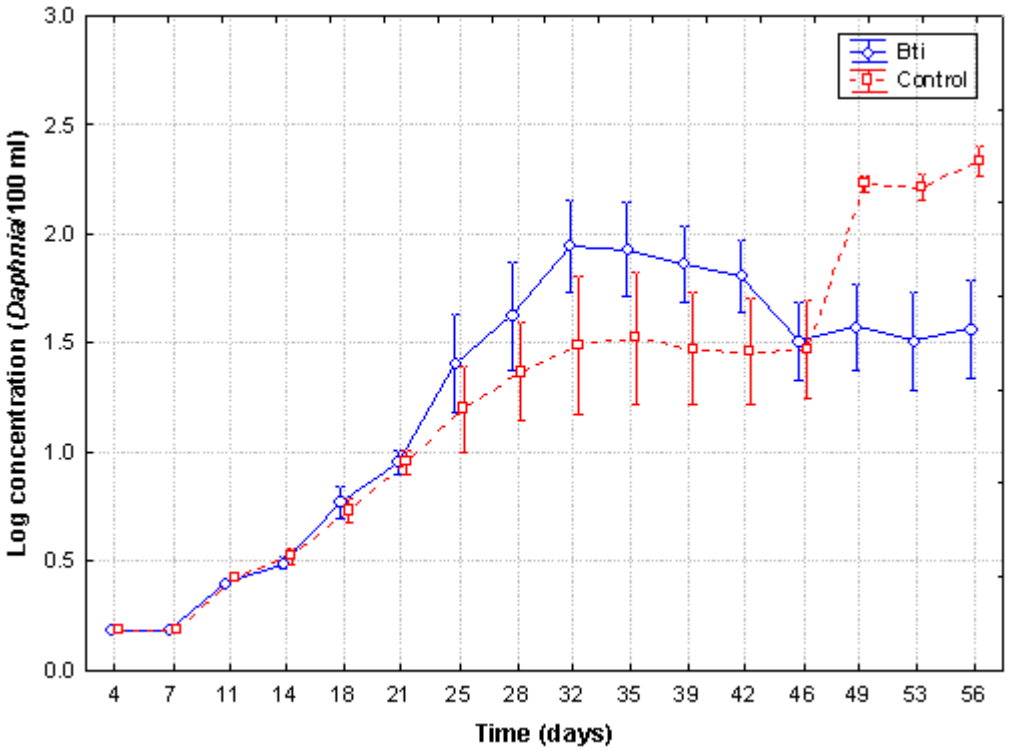

Figure 4.5 Change in concentration of *Daphnia pulex* populations in control and *Bti* microcosms during the 56-day experiment. Values shown are mean ± SE, n=6 for each data point.

Although the *C. africanus* populations in the control and *Bti* microcosms had different profiles (Figure 4.6), the main effect of treatment was not significant ($F_{1,10}$=0.2988; p=0.5966). The interaction between time and treatment was also statistically not significant ($F_{15,150}$=0.5167; p=0.9285). *C. africanus* populations exhibited a long lag phase until day 14 (Figure 4.6). *C. africanus,* in the *Bti* and control microcosms, did not appear to flourish as a maximum log concentration of 0.55/100 ml was recorded by the end of the 56 day period.

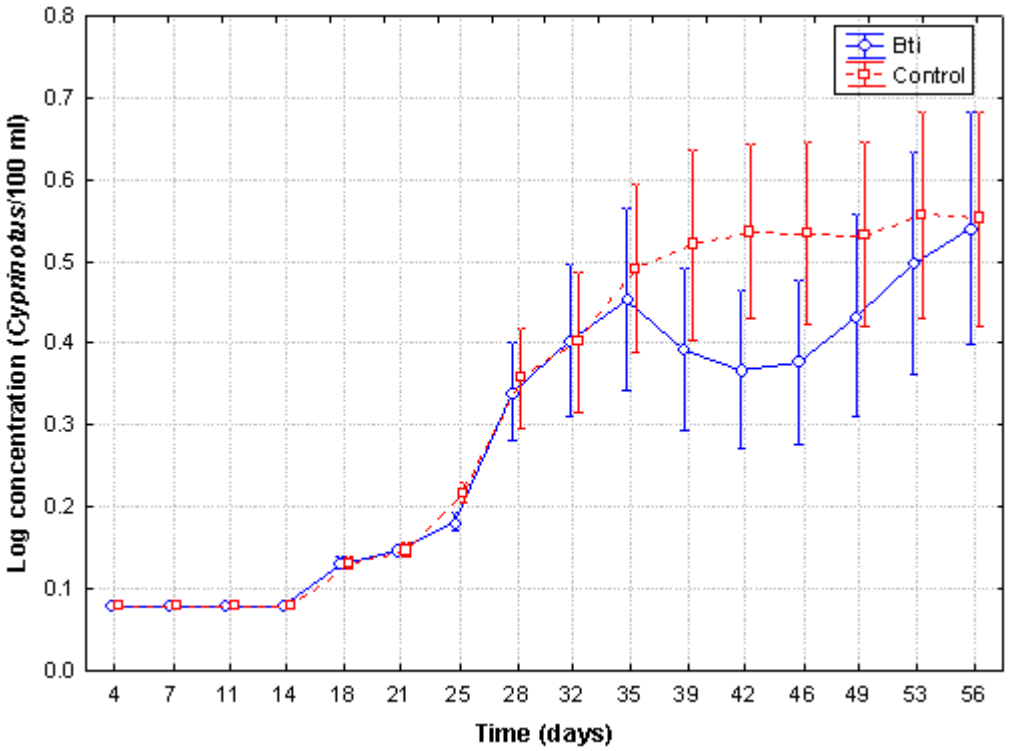

Figure 4.6 Change in concentration of *Cyprinotus africanus* populations in control and *Bti* microcosms during the 56-day experiment. Values shown are mean ± SE, n=6 for each data point.

The concentration of *Lepadella* sp. populations increased gradually from day 7 until day 56 (Figure 4.7), with near-exponential increases in population concentrations. The interaction between time and treatment was statistically not significant ($F_{15,150}$=0.1122; p=1.000). The main effect of treatment was also not significant ($F_{1,10}$=0.0227; p=0.8834).

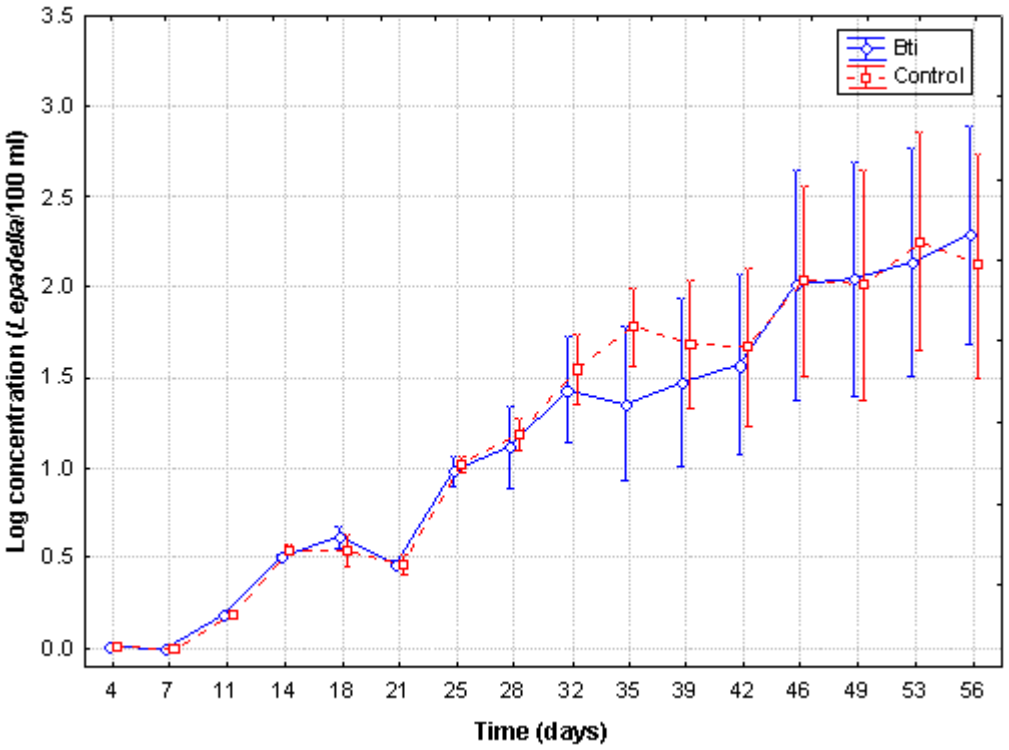

Figure 4.7 Change in the concentration of *Lepadella* sp. populations in control and *Bti* microcosms during the 56-day experiment. Values shown are mean ± SE, n=6 for each data point.

The change over time in the concentration of *Coleps* sp. populations was similar in the *Bti* and control microcosms (Figure 4.8). The interaction between time and treatment was not significant ($F_{15,150}$=0.5483; p=0.9090), as was the main effect of treatment ($F_{1,10}$=0.0104; p=0.9207). The *Coleps* sp. populations were established very quickly, increasing rapidly to log concentrations of approximately 3/100 ml by day 21 (Figure 4.8). Populations decreased after day 21 and increased again after day 42 (Figure 4.8).

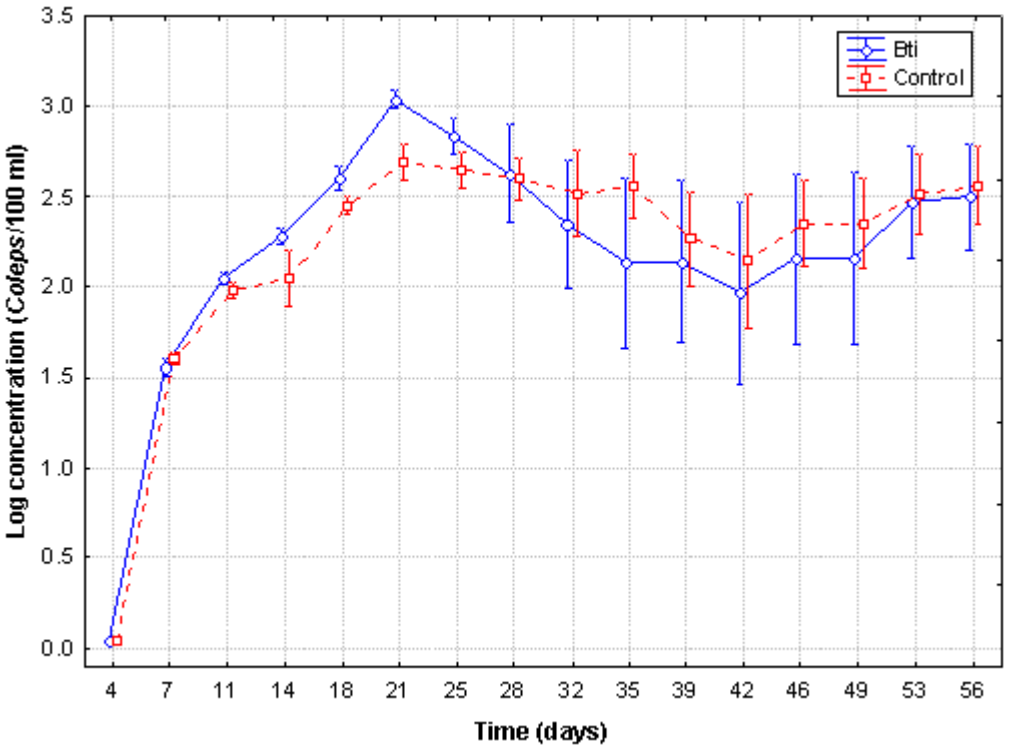

Figure 4.8 Change in concentration of *Coleps* sp. populations in control and *Bti* microcosms during the 56-day experiment. Values shown are mean ± SE, n=6 for each data point.

Oxygen gain or photosynthesis increased gradually until day 28 (Figure 4.9), corresponding to an increase in PMO concentration (Figure 4.4). From day 28, there was a decrease in the net photosynthesis (Figure 4.9). The main effect of treatment was not significant ($F_{1,10}$=0.969; p=0.7619) and the interaction between treatment and time was statistically not significant ($F_{15,150}$=0.4355; p=0.9662).

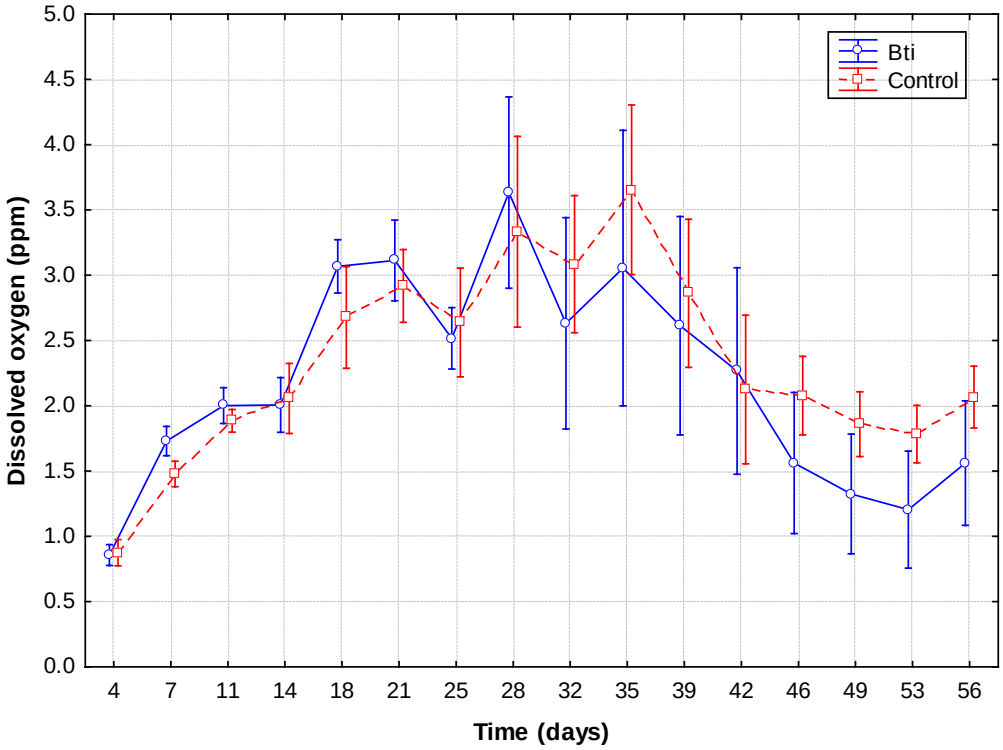

Figure 4.9 Change in net photosynthesis in control and *Bti* microcosms during the 56-day experiment. Values shown are mean ± SE, n=6 for each data point.

4.5 Discussion

Bti was added to the experimental microcosms on days 7, 21, 35, and 49. Twenty-four hours p.i. (day 8), the *An. arabiensis* larvae were reduced by 87%, however 7 days p.i. larval mortality was reduced by only 15%. This suggests that *Bti* did not persist for more than 7 days, as has been found in previous studies (Mulligan *et al.*, 1980; Davidson *et al*., 1981; Manasherob *et al*., 2003; Zahiri *et al*., 2004). Although several environmental factors have been known to affect the larvicidal activity of *Bti*, in the SAM experiment the environmental factors such as sunlight (UV), water quality, amounts and composition of food are standardized. Thus the major factors that could influence the observed larvicidal activity of *Bti* in our study are differences in other filter feeding organisms competing for food, and rapid settling of the *Bti* spores and crystals which may limit the contact with larval populations (Ignoffo & Garcia, 1978; Ignoffo *et al*., 1981; Mulla *et al*., 1990; Blaustein & Margalit, 1991; Becker *et al*., 1992; Sheeran & Fisher, 1992).

In situations where no agitation occurs, *Bti* tends to settle from the surface of the water, reducing larvicidal activity especially if the mosquito larvae are surface-feeders (like *An. arabiensis*) (Ignoffo *et al*., 1981; Mulla *et al*., 1982; Merritt *et al*., 1992). Studies have also reported that although *Bti* is likely to adsorb to the sediment and particulates, the *Bti* spores and crystals (referred to as particles) are released back into the water with agitation (Ohana *et al*., 1987; Sheeran & Fisher, 1992). In our study, the *An. arabiensis* larvae were added to microcosms that had been agitated so any settled or bound *Bti* particles would have been resuspended. However, larvicidal activity was still greatly reduced 7 days p.i., suggesting that factors other than agitation played a role in the larvicidal activity and persistence of *Bti* in these microcosms.

The larvicidal activity of *Bti* can also be adversely affected by the availability of other food sources in the environment. The larvicidal activity of *Bti* was 22% lower after addition on day 21 than after addition on day 7, suggesting that as the microcosm develops *Bti* larvicidal activity decreases. When *Bti* was initially added to the microcosms, the PMO concentrations were low. This suggests that the *An. arabiensis* larvae may not have had any other food sources readily available, thus more *Bti* was ingested resulting in high larval mortality. Mosquito larvae have been shown to ingest algae (Thiery *et al*., 1991; Stevens *et al*., 1994 Marten, 2007), and in this study the midgut region of the *An. arabiensis* larvae appeared green showing that the larvae were feeding on the PMOs. The presence of alternative food sources in the midgut of the *An. arabiensis* larvae could limit or prevent any ingested *Bti* particles from binding to the specific midgut receptors (Ali *et al*., 1981; Aly, 1983; Hofmann *et al*., 1988; Ravoahangimalala *et al*., 1993; Ben-Dov *et al*., 2003). Alternatively, if less *Bti* is ingested larval mortality may be reduced, as there is less *Bti* to bind to the midgut receptors.

The decrease in larvicidal activity could also be due to the fact that all the invertebrates evaluated in this study could act as competitors by grazing on

bacteria and other microscopic particles, with the main competitor being *D. pulex* (Peterson *et al*., 1978; Blaustein & Margalit, 1991, Becker *et al*., 1992; Sterner & Hessen, 1994; Hegg, 2002; Duchet *et al*., 2008; Forró *et al*., 2008). Ostracods have also been shown to ingest *Bti* and it has been suggested that they decrease the concentration of *Bti* in the water, reducing larval mortality (Blaustein & Margalit, 1991; Brain, 2002). Initial concentrations of *D. pulex* populations were probably not high enough to compete with the *An. arabiensis* larvae for the *Bti* particles. This suggests that there would be a high concentration of *Bti* particles available for the *An. arabiensis* larvae, resulting in high mortality. However, as the *D. pulex* populations increased in concentration, competition for the *Bti* particles is likely to increase. Furthermore, most *Daphnia* sp. are filter feeders and also tend to feed on bottom sediments so any settled *Bti* could have been ingested (Blaustein & Margalit, 1991; Hessen *et al*., 2000). Thus, as the invertebrate populations in the microcosms increase (from day 14), the grazing on bacteria and *Bti* particles is likely to increase, possibly reducing the availability of *Bti* for the mosquito larvae, and subsequently reducing *An. arabiensis* larval mortality.

Bti was shown to be non-toxic to the invertebrates evaluated in this study and did not appear to effect any of the other variables such as water quality or inorganic nutrients in the microcosms. This compares to the findings of other research groups that found *Bti* was not toxic to non-target aquatic organisms in both laboratory and field studies (Ali, 1981; Kreutzweiser *et al*., 1996; Hershey *et al*., 1998; Milam *et al*., 2000). The trends for all the variables evaluated, in the control and *Bti* microcosms, were similar. The main effect of treatment on all the variables was not significant. However, the interaction between time and treatment was shown to be significant only on day 56 by *post hoc* Bonferroni tests in the *D. pulex* populations.

Nutrient availability seemed to follow a natural course with the nitrates, nitrites, and phosphates being utilized and depleted by day 25 (Taub *et al*.,

1988; Taub, 1989; Taub *et al*., 1991; Meador *et al*., 1993; Tsirtsis & Karydis, 1996). These nutrients (nitrates, nitrites, phosphates) have been previously shown to affect primary production (PMO growth) (Sterner & Hessen, 1994). As seen in this study and others of a similar nature, the increase in PMO concentration was coupled with a decrease in inorganic nutrients (Taub *et al*., 1988; Taub *et al*., 1991; Meador *et al*., 1993; Tsirtsis & Karydis, 1996). The PMOs utilize the available nutrients, converting the inorganic chemicals to organic chemicals for growth (Taub, 2009). Once the nitrates, nitrites, and phosphates are depleted, PMOs rely on the recycled phosphate (and ammonia) to maintain their populations, in spite of losses due to grazing (Fallon & Brock, 1979). The increase in the concentration of phosphate observed after day 28 could be a result of the phosphate that has been recycled (Taub *et al*., 1991).

Ammonia is a waste product and as the number of invertebrates increased so did the concentration of ammonia, as was expected (Tsirtsis & Karydis, 1996). In this study, the presence of *Bti* did not appear to influence the concentration of ammonia in the microcosms as there was no significant difference in ammonia concentration between the control and *Bti* microcosms.

The PMOs are important for producing oxygen (Taub, 2009) and for providing nutrients for the invertebrates. In this study, there was a corresponding increase in DO (presented as photosynthesis) as the PMO concentrations increased. This was also found in other studies (Taub *et al*., 1991; Clément & Cadier, 1998). As PMO concentrations decreased, as a result of either an increase in *D. pulex* grazing (reducing PMO concentrations) and/or nutrient depletion, the DO also decreased. Dissolved oxygen concentrations below 1.0 ppm are lethal to *Daphnia* sp. (Taub, 2009), however the DO concentrations remained above 1.0 ppm, in the control and *Bti* microcosms, throughout the study. As shown in this study, the addition of *Bti* to the microcosms did not significantly affect the DO levels, PMO concentrations or inorganic nutrients,

as there were no statistically significant differences in these variables between the control and *Bti* microcosms.

The inorganic nutrients present in the microcosms, indirectly affect the growth and development of the grazer or invertebrate populations (Sterner, 1993). The grazers such as *D. pulex*, *C. africanus*, *Coleps* sp., and *Lepadella* sp. rely on PMO development for their development; hence without the nutrients required for PMO production, the grazers' development would be slow (Sterner, 1993). As shown in this study, as the PMO populations increased, the number of invertebrates increased, although the most obvious affect of changes in PMO populations was the changes in the *D. pulex* population concentrations. As reported in similar microcosm studies (Taub *et al*., 1988; Meador *et al*., 1993; Barry & Logan, 1998; Clément & Cadier, 1998), as the *D. pulex* populations increased, the PMOs available for feeding decreased.

Initially, the rate of *D. pulex* population increase depends on the number of eggs carried by the inoculated adults, which is likely to affect their early clutch size (McCauley *et al*., 1990). The number of eggs carried by the inoculated adults was not standardized in this study, as standardization of egg number is not indicated in the protocol (ASTM, 2002). Hence, the number of first off-spring was likely to vary resulting in differences in the population numbers (Taub, 1993) (as indicated by the relatively large error bars) in the different replicates of the treatments. Only three adults with eggs were introduced into each microcosm (as per the ASTM protocol). The first clutch size would thus depend on the number of eggs carried by these adults (normally ranges from 4 to 22 eggs per clutch) (McCauley *et al*., 1990). If the adult carried few eggs, then the first clutch would be small and subsequent populations would experience a lag in growth, which appears to have occurred in some of the control microcosms.

The plateau observed in *D. pulex* control populations between days 32 and 46 could have been a result of unfavourable conditions such as lack of food, low

oxygen supply, or low temperatures (McCauley *et al*., 1990; Gliwicz & Guisande, 1992; Guisande & Gliwicz, 1992). Under these conditions, in particular low food supply, the *Daphnia* sp. decrease their clutch size (Guisande & Gliwicz, 1992). Towards the end of the 56 day period, the dominant PMO species were *A. cylindrical* and the *Ankistrodesmus* sp. and not the more favourable unicellular PMOs like *C. vulgaris*, *S. capricornutum*, and *S. obliquus* (Thompson *et al*., 1982; Taub, 1989). The dominant PMO species may have been unacceptable food sources for *D. pulex*, thereby prompting a reduction in clutch size.

The *Bti* microcosms initially had higher (not statistically higher) *D. pulex* populations than the control microcosms and at day 32 started to decrease, at the same time that the *D. pulex* populations in the control microcosms were starting to plateau. The decline (observed in *Bti* microcosms; day 32) and plateau (observed in the control microcosms; day 32) in *D. pulex* populations could have been caused by a decrease in PMO populations between days 28 and 46, and the rapid decrease in DO from day 35 to day 46.

Statistically, however, there was no significant difference between the control and *Bti* microcosms except for day 56, as shown by *post hoc* tests. This difference however, cannot be attributed to the presence of *Bti*, as a significant difference was only observed at the end of the experiment and so direct effects of *Bti* are very unlikely (Taub *et al*., 1980; Duchet *et al*., 2008). If *Bti* had a significant effect on *D. pulex* populations, the differences in the trends in *D. pulex* populations should have occurred much earlier as has been shown in other studies where the treatment effect occurs within days of application of the toxin or chemical (Taub *et al*., 1988; Taub *et al*., 1991; Sugiura, 1992; Meador *et al*., 1993), rather than only at the end of a 56 day period. This study confirms the findings of previous studies whereby *Bti* was shown to have no effect on *D. pulex* populations (Milam *et al*., 2000; Duchet *et al*., 2008).

The concentration of *C. africanus* and *Lepadella* sp. populations increased up until day 56 with *C. africanus* populations remaining low, as seen in other studies (Taub *et al*., 1980; Kindig *et al*., 1983; Diner *et al*., 1986). The presence of *Bti* did not affect the *C. africanus* populations, as there was no significant difference between the populations in the control and *Bti* microcosms. It has previously been shown that some *Daphnia* sp. kill rotifers by exploitative competition for shared, limited resources and through mechanical interference (Gilbert, 1985; Conde-Porcuna, 1998). However, in this study, the presence of *D. pulex* in control and *Bti* microcosms did not appear to affect the *Lepadella* sp. populations. Importantly, the presence of *Bti* did not have a statistically significant effect on the *Lepadella* sp. population concentrations.

The trends in the *Coleps* sp. growth were different to those of *D. pulex*, *Lepadella* sp., and *C. africanus*. The *Coleps* sp. population concentrations increased before the other invertebrates and then decreased as *D. pulex* populations increased and PMO concentration decreased. The decrease in protozoans by day 21 could also be attributed to the fact that by day 21, the larger *D. pulex* could act as predators of protozoans (Pace & Vaqué, 1994), reducing their numbers and keeping their numbers lower than the peak on day 21. However, there was no significant effect of *Bti* on the protozoan populations, as the protozoans exhibited statistically similar profiles in the control and *Bti* microcosms.

The effect of *Bti* on target and non-target organisms was evaluated in this study. Although numerous studies have reported the effect of *Bti* on non-target and target organisms, experimental designs and methodologies are different and could lead to different conclusions with regards to the effect of *Bti* on target and non-target species (Boisvert & Boisvert, 2000; Milam *et al*., 2000; De Melo-Santos *et al*., 2009). To our knowledge, this is the first study using the SAM protocol to evaluate the effects of *Bti* on non-target organisms.

The SAM protocol enables evaluation of the effects of *Bti* on non-target organisms (and other variables) in a comprehensive, reproducible, and cost-effective manner thereby removing many uncertainties with regards to the effect of *Bti* on non-target organisms that may currently exist. The SAM protocol provides a good indication of the interactions between the trophic levels, which can be used to predict the interactions in similar types of communities in the field, giving the SAM experiment an advantage over single-species testing.

Bti larvicidal persistence and activity was reduced significantly a week after inoculation. The reduction of larvicidal activity as the PMO and invertebrates increased in the microcosms, suggests that environmental conditions like high concentrations of PMOs or the presence of potential invertebrate competitors could reduce the larvicidal activity of *Bti*. The general trends observed in the control and *Bti* microcosms in this study were comparable to the control microcosms of other SAM studies that were used to evaluate the effect of other chemical compounds in the aquatic environment (Taub *et al*., 1988; Taub *et al*., 1991; Meador *et al*., 1993).

In addition to a reduction in *Bti* larvicidal activity against the target organism, our microcosm study also showed that the concentration (an LC_{90}) of *Bti* added did not have any significant effects on the non-target invertebrates or other variables (e.g. water quality) evaluated, showing that *Bti* is an environmentally friendly larvicidal agent. The SAM protocol used in this study could provide a foundation for future comparisons of the non-target effects of *Bti* and other biopesticides.

Chapter 5

Evaluation of non-target effects and larvicidal persistence of a genetically engineered cyanobacterium, *Anabaena* PCC 7120#11, in a Standardized Aquatic Microcosm

5.1 Abstract

The cyanobacterium, *Anabaena* PCC 7120, had previously been genetically engineered to act as a delivery vehicle for *Bacillus thuringiensis* subspecies *israelensis* (*Bti*) toxins. In previous laboratory bioassays, the genetically engineered cyanobacterium, *Anabaena* PCC 7120#11 (PCC 7120#11), was found to be highly larvicidal against several important vectors of malaria such as *Anopheles arabiensis*. The non-target effects and larvicidal persistence of PCC 7120#11 in the field needs to be evaluated, especially since there are concerns with releasing genetically engineered microorganisms into the environment. The objective of this study was to use a multi-species test, the Standardized Aquatic Microcosm, to evaluate the non-target effects and larvicidal persistence of PCC 7120#11. The microcosms are initiated with the same chemically defined medium and sediment, same species and concentration of photosynthetic microorganisms (PMOs) and invertebrate species. At a concentration of 4.5 x 10^6 cells/ml, the larvidical activity of PCC 7120#11 against *An. arabiensis* larvae was reduced from an initial mortality of 72.3 ± 4.50% to 17.7 ± 8.29%, within 7 days. Changes in the amount and composition of available food sources could have reduced the larvicidal activity of PCC 7120#11. Treatment, PCC 7120#11 or control, was not significant as a main effect for several of the evaluated variables. However, the main effect of treatment and the interaction between treatment and time was significant for total PMO cell concentration, nitrate and phosphate concentrations. Decomposition of the PCC 7120#11 cells could have lead to the increased phosphate and nitrate concentrations observed on specific days. The interaction between time and treatment was significant for three of the invertebrate populations evaluated. This study showed that PCC 7120#11 did not persist in high concentrations in the microcosms suggesting that it will not persist in the natural environment. Furthermore, by the end of the experiment there was no significant differences in the PCC 7120#11 microcosms for any of the variables evaluated, suggesting that PCC 7120#11 only had a temporary effect. This study shows that PCC 7120#11 is thus

unlikely to have a long-term effect on non-target organisms in the field, and demonstrates the potential of PCC 7120#11 to be an environmentally friendly malaria vector control agent.

5.2 Introduction

Bacillus thuringiensis subspecies *israelensis* (*Bti*) has been used as a larvicidal agent for several years against several mosquito species belonging to the genera *Culex*, *Aedes*, and *Anopheles* (Tyrell *et al*., 1979; Van Essen & Hembree, 1980; Ignoffo *et al*., 1981; Seyoum & Abate, 1997; Zahiri *et al*., 2004). *Bti* is a spore-forming, Gram-positive, soil bacterium that produces insecticidal crystalline inclusions that contain highly toxic Cry (crystal) and Cyt (cytolytic) proteins (Schnepf *et al*., 1998). However, there are several limitations to using *Bti* as a larvicidal agent. These include its low persistence in the field due to the *Bti* spores settling out of the mosquito larval feeding zone, adsorption to particles, inactivation of the *Bti* spores from UV light, and lack of replication of the spores in the aquatic environment (Griego & Spence, 1978; Ignoffo *et al*., 1981; Ramoska, 1982; Ohana *et al*., 1987; Becker *et al*., 1992; Boussiba *et al*., 2000; Manasherob *et al*., 2003). Although *Bti* and its formulations have been moderately successful, they do not exhibit long term larvicidal activity in the environment and would thus require weekly additions for sustained vector control (Federici *et al*., 2003; Fillinger *et al*., 2003; Mittal, 2003; Gunasekaran *et al*., 2004). In an attempt to overcome the limitations of *Bti* as a larvicidal agent, several microorganisms have been genetically engineered as potential larvicidal agents (Yap *et al*., 1994a; Liu *et al*., 1996; Khampang *et al*., 1999; Boussiba *et al*., 2000; Tanapongpipat *et al*., 2003; Armengol *et al*., 2005).

The cyanobacterium, *Anabaena* sp. strain PCC 7120 (hereafter referred to as PCC 7120), is a filamentous, nitrogen-fixing cyanobacterium capable of multiplying in mosquito breeding sites, maintaining its position in the water column, and can resist a range of environmental conditions (Boussiba *et al*.,

2000; Manasherob *et al*., 2002; Manasherob *et al*., 2003), thereby making it a suitable candidate for genetic engineering for improved mosquito control. Furthermore, unlike other species of *Anabaena*, PCC 7120 does not produce neuromuscular toxins and is unlikely to affect humans or animals (Kurmayer & Jüttner, 1999). As a result of its suitable characteristics, PCC 7120 was genetically engineered to act as a delivery vehicle for *Bti* toxins. The *cry 4Aa*, *cry 11Aa* genes from *Bti* were introduced together with an accessory protein gene, *p20*, into PCC 7120 using an *Escherichia coli-Anabaena* shuttle vector pRL488p (Wu *et al*., 1997). The resultant clone, *Anabaena* PCC 7120#11, was found to be highly larvicidal to *Aedes aegypti* (Wu *et al*., 1997; Manasherob *et al*., 2003) and several important anopheline species, tested to date (Chapter 3).

In laboratory studies, important malaria vectors such as *Anopheles arabiensis* and *Anopheles gambiae* were shown to be susceptible to *Anabaena* PCC 7120#11 (hereafter referred to as PCC 7120#11) (Chapter 3), however the non-target effects and persistence of PCC 7120#11 as a larvicidal agent in the field needs to be evaluated (Hershey *et al*., 1998). There are concerns and potential risks with releasing genetically engineered microorganisms (GEMs) in the field (Williamson, 1992; Porter *et al*., 1993; Stephenson & Warnes, 1996; Giddings, 1998; Kaur, 2000). The unknown effects on beneficial non-target species and fauna, transfer of transgenes to other species, and potential detrimental long-term effects are the main risks associated with environmental releases (Kimball & Levin, 1985; Porter, 1996; Stephenson & Warnes, 1996; Giddings, 1998; Boussiba *et al*., 2000; Wolfenbarger & Phifer, 2000). Due to the concerns of testing PCC 7120#11 in the field and the difficulty in obtaining regulatory approval for field trials of GEMs, laboratory microcosms are the most viable method of addressing some of the concerns associated with GEMs that are intended for application to water bodies (Krimsky *et al*., 1995; Crane, 1997).

Synthetic microcosms that resemble the environment have been developed, with certain properties of the environment being selected and others excluded (Krimsky *et al*., 1995). There are several methods that allow researchers to study potential environmental risks namely single-species tests, two or three species tests, gnotobiotic ecosystems, and complex multi-species tests (Taub, 1992). Of particular interest in this study was the use of a multi-species test, the Standardized Aquatic Microcosm (SAM) experiment, to evaluate the non-target effects and larvicidal persistence of PCC 7120#11.

The SAM is a small, simple and reliable system that mimics a generalized aquatic community, with important ecological such as primary and secondary production being demonstrated by the SAM experiment (Taub, 1989; Meador *et al*., 1993). The microcosms are standardized in that the experiment is initiated with the same chemically defined medium and sediment, same species and concentration of photosynthetic microorganisms (PMOs) such as algae and cyanobacteria, and invertebrate species (Taub, 1989; Taub, 1997). This allows for repeatability within a laboratory and between laboratories (Taub, 1997). Although the SAM experiment is more complex than single-species tests, it is not as complex as a natural environment thus, reducing the likelihood that taxonomic uncertainties will occur (Taub, 1997). Another advantage of the SAM protocol is that the experiments can be conducted and initiated at any time of the year (Taub, 1997). This is the first study, to our knowledge, that allowed the environmentally safe evaluation of the effects of PCC 7120#11 on non-target organisms and a target malaria vector. With regards to larvicidal activity, the target organism is the malaria vector, *An. arabiensis* which is one of the seven recognized species belonging to the *An. gambiae* complex (Coetzee *et al*., 2000). *An. arabiensis* is the most widespread vector of malaria in southern Africa (Coetzee *et al*., 2000), and thus its control is critical. This study provided valuable information on the effects of PCC 7120#11 on the non-target organisms, the persistence of the

PCC 7120#11 cells, and the larvicidal persistence of PCC 7120#11 on the target organism, in an aquatic environment.

5.3 Methodology

5.3.1 Microcosms

The microcosms were set up according to the SAM protocol as described in the ASTM E1366-02 Standard Practice for Standardized Aquatic Microcosms: Fresh Water (ASTM, 2002), with conditions and modifications as previously described in the Methodology section of Chapter 4 under the subheading of "Microcosms". Six jars (replicates) were allocated for PCC 7120#11 and six jars (replicates) for the control, with an additional five prepared in case of breakages within the first week (ASTM, 2002).

5.3.2 Culturing of PMOs

The nine algal and cyanobacterial species (hereafter referred to as PMOs); *Anabaena cylindrica*, *Ankistrodesmus* sp., *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Oscillatoria simplicissima*, *Scenedesmus obliquus*, *Selenastrum capricornutum*, *Stigeoclonium* sp., and *Ulothrix* sp., were cultured and added to the microcosms as previously described in the Methodology section of Chapter 4 under the subheading "Culturing of PMOs". The counts were calculated using the equation presented in the ASTM protocol (2002).

5.3.3 Culturing of invertebrates

The cladocerans (*Daphnia pulex),* the ostracods (*Cyprinotus africanus*), the protozoans (*Coleps* sp.), and the rotifers (*Lepadella* sp.) were cultured and added to the microcosms as described previously in the Methodology section of Chapter 4 under the subheading "Culturing of invertebrates".

5.3.4 Culturing of PCC 7120#11

The genetically engineered PCC 7120#11, was cultured in BG-11 medium (Wu *et al*., 1997), at 30 °C under continuous illumination (2000 lux) with constant agitation on a rotary shaker at 140 revolutions per minutes (Lluisma *et al*., 2001; Manasherob *et al*., 2003), until mid-exponential growth phase.

5.3.5 Determination of the concentration PCC 7120#11

The cells were harvested by differential centrifugation at 10 000 *g* (Beckman J2-21 with JA-14 rotor, Beckman Coulter, U.K.) for 25 minutes at 15 °C. The pellet was washed twice and suspended in a minimal volume of sterile distilled water. The cell concentration was determined by using a Neubauer haemocytometer (Superior Co., Berlin, Germany). A concentration of 4.5×10^6 cells/ml, resulting in 90% mortality (LC_{90}) of *An. arabiensis* larvae, was added, only once (single addition), to six of the microcosm jars on day 7 of the experiment.

5.3.6 Mosquito species

An. arabiensis (origin and colony name provided) (Kanyemba, Zimbabwe; KGB), was obtained from colonies maintained at the National Institute of Communicable Diseases in Johannesburg, South Africa. The larvae were reared in a controlled environment of 25 °C, relative humidity of 60%, and a L:D 12:12 hour photoperiod with a 45 minute dawn and dusk light regime. Thirty third instar mosquito larvae were added to the PCC 7120#11 and control microcosm jars on day 7. The surviving larvae were counted 24 hours post-inoculation (p.i.), after which all larvae were removed from the microcosms. Larvae were added to all the microcosms weekly and mortality recorded 24 hours p.i.

5.3.7 Sample collection

Sampling and measurement of organism abundances and physical factors were performed twice a week on the same days of the week (Tuesdays and

Fridays). The samples were collected and analysed as described in Chapter 4 in the Methodology section under the subheading of “Sample collection”.

5.3.8 Chemical analysis

The chemical analysis of the filtered microcosm water was performed as described in Chapter 4 in the Methodology section under the subheading of “Chemical analysis”. The samples were all processed on the sampling day.

5.3.9 Statistical analysis

A repeated measure analysis of variance (rm-ANOVA) with a between-subject effect (treatment) and a within-subject effect (time) was used to assess the effects of the treatment on each variable evaluated. The statistical analysis was performed as described in Chapter 4 in the Methodology section under the subheading of “Statistical analysis”. Differences were considered significant at $\alpha = 0.05$ for all tests.

5.4 Results

5.4.1 Target effects

A single application of PCC 7120#11 was added to six microcosm jars on day 7, at a concentration that, on the basis of pre-SAM ranging assays should have resulted in 90% mortality (i.e. LC_{90}) of the third instar *An. arabiensis* larvae. Subsequently, larvae were added to the microcosms on days 14, 21, 28, and 35 with larval mortality recorded 24 hours p.i. After the initial addition of PCC 7120#11, *An. arabiensis* larval mortality on day 8 was 72.3 ± 4.50% (mean values ± SE; n=180; 6 replicates of 30 larvae per replicate). A week later (day 15), larval mortality dropped to 17.7 ± 8.29%. On the remaining evaluation days (22, 29, and 36), no mortality of the *An. arabiensis* larvae was observed. No larval mortality was recorded in any of the control microcosms.

5.4.2 Effects on variables

The net photosynthesis or net oxygen gain increased from day 4 until day 18 and then decreased continuously until day 35 (Figure 5.1). At the end of the 35 day period, the net photosynthesis in the PCC 7120#11 microcosms was slightly above 0 ppm and above 1 ppm in the control microcosms (Figure 5.1). The main effect of treatment on photosynthesis was not significant ($F_{1,10}$=0.2623; p=0.6170). The interaction between treatment and time was also not statistically significant ($F_{9,90}$=1.395; p=0.2021). As expected, the main effect of time was statistically significant for all the variables (physical, chemical, and invertebrates) evaluated and will thus not be mentioned further.

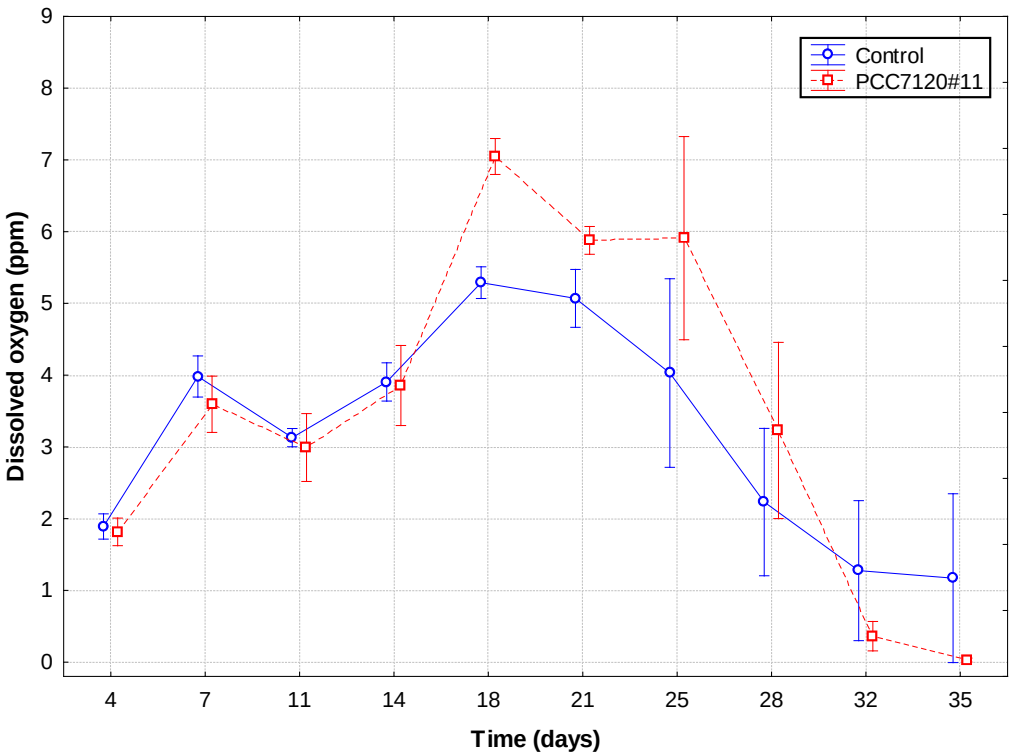

Figure 5.1 Change in the net photosynthesis in control and PCC 7120#11 microcosms during the 35-day period. Values shown are mean value ± SE, n=6 for each data point.

The nitrate concentration decreased from an initial value (day 0) of 7 mg/l to below 1 mg/l by day 35 (Figure 5.2). The change in nitrate concentration over time in both the control and PCC 7120#11 microcosms was similar (Figure 5.2), however the main effect of treatment was found to be statistically

significant ($F_{1,10}$=29.09; p=0.0005). The interaction between time and treatment was also found to be statistically significant ($F_{9,90}$=2.390; p=0.0180). *Post hoc* Bonferroni tests showed that the significant difference only occurred on day 18, with the nitrate concentration being lower (0.5 mg/l) in the control microcosms than in the PCC 7120#11 microcosms (approximately 1.8 mg/l) (Figure 5.2).

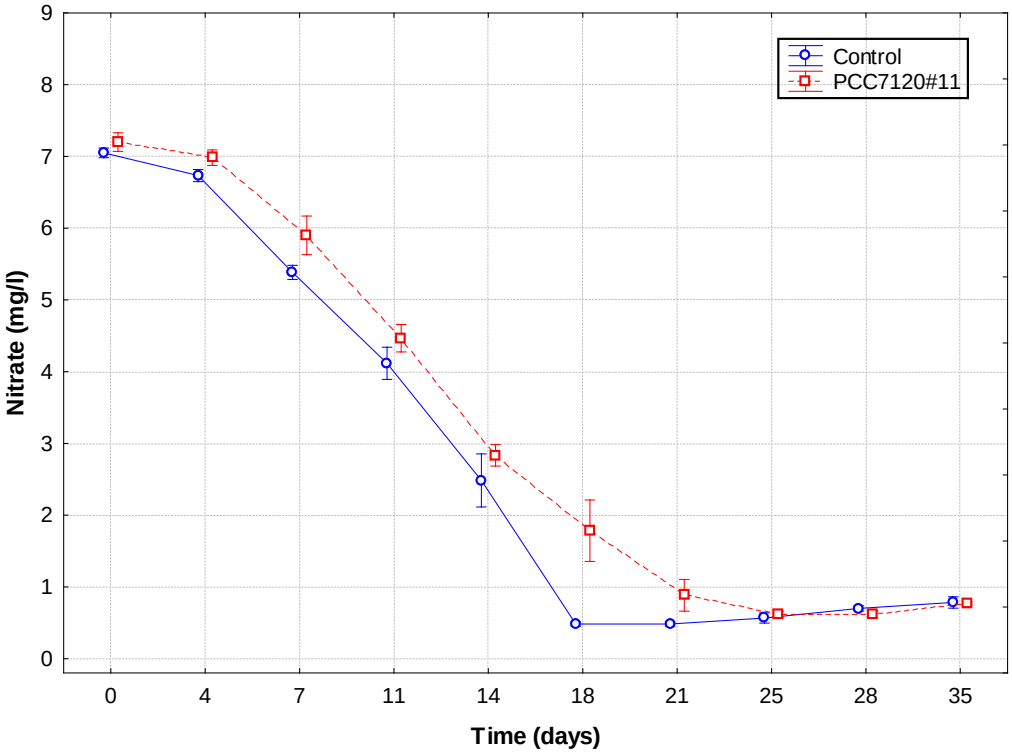

Figure 5.2 Change in nitrate concentration in control and PCC 7120#11 microcosms during the 35-day period. Values shown are mean value ± SE, n=6 for each data point.

The change in nitrite concentration over time in the PCC 7120#11 microcosms was similar to the change in nitrite concentration over time in the control microcosms (Figure 5.3). Initial nitrite concentrations were found to be approximately 0.65 mg/l, decreasing rapidly to almost undetectable limits (slightly above 0 mg/l) by day 18 (Figure 5.3). The nitrite concentration remained low until the end of the experiment (Figure 5.3). The interaction between time and treatment was not significant ($F_{9,90}$=0.4790; p=0.8851), as was the main effect of treatment ($F_{1,10}$=0.080; p=0.7826).

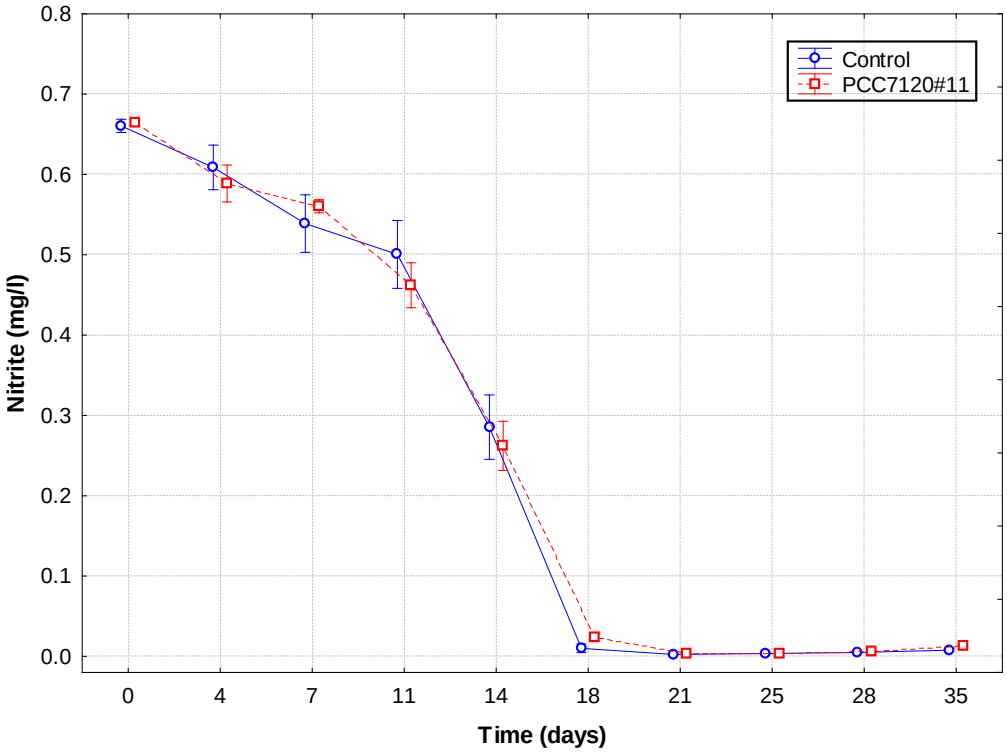

Figure 5.3 Change in nitrite concentration in control and PCC 7120#11 microcosms during the 35-day period. Values shown are mean value ± SE, n=6 for each data point.

In the control and PCC 7120#11 microcosms, the initial concentration of phosphate was approximately 1.8 mg/l (Figure 5.4). The phosphate concentration decreased in the control and PCC 7120#11 microcosms, with the phosphate concentration decreasing more rapidly to approximately 0.2 mg/l by day 18 in the control microcosm, whereas 0.2 mg/l was reached by day 25 in the PCC 7120#11 microcosms (Figure 5.4). By day 35, there was an increase in phosphate concentration in the control microcosms to approximately 0.8 mg/l and an increase to approximately 1.0 mg/l in the PCC 7120#11 microcosms (Figure 5.4). The main effect of treatment on phosphate concentration was statistically significant ($F_{1,10}$=18.15; p=0.002). The interaction between time and treatment was also statistically significant ($F_{9,90}$=4.420; p<0.01). *Post hoc* tests showed that the phosphate concentration was significantly higher only on days 14 and 18, in the PCC 7120#11 microcosms than in the control microcosms (Figure 5.4).

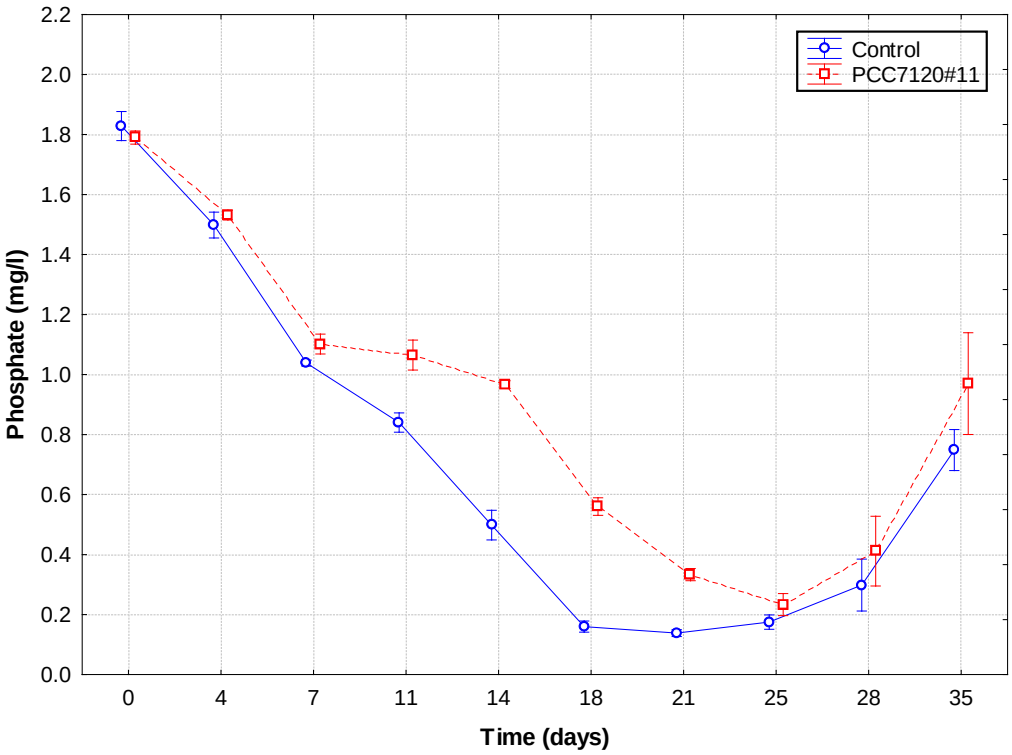

Figure 5.4 Change in phosphate concentration in control and PCC 7120#11 microcosms during the 35-day period. Values shown are mean value ± SE, n=6 for each data point.

Initial concentrations of ammonia were slightly above 0 mg/l in the PCC 7120#11 and control microcosms (Figure 5.5). The concentration of ammonia increased rapidly after day 28 reaching concentrations of approximately 2.0 mg/l by day 35 (Figure 5.5). The main effect of treatment on ammonia concentration was not significant ($F_{1,10}$=0.0315; p=0.8626). The interaction between treatment and time was also statistically not significant ($F_{9,90}$=0.5399; p=0.8418).

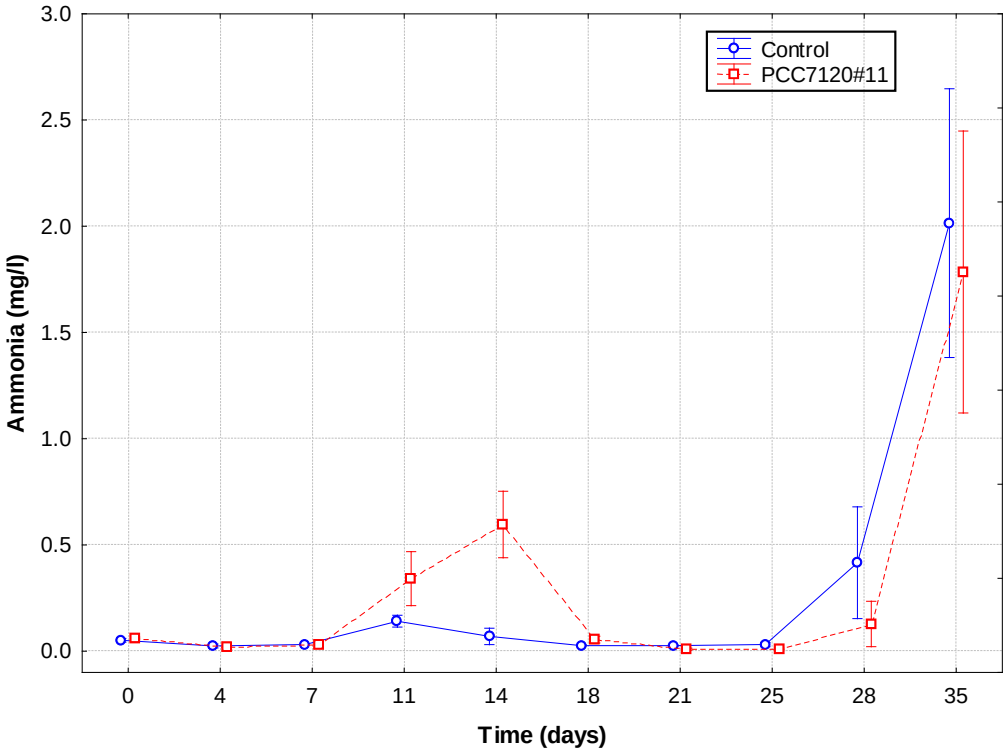

Figure 5.5 Change in ammonia concentration in control and PCC 7120#11 microcosms during the 35-day period. Values shown are mean value ± SE, n=6 for each data point.

In the control and PCC 7120#11 microcosms, the log transformed total PMO cell concentration increased rapidly from day 0 to day 4 (Figure 5.6). After the addition of PCC 7120#11 on day 7, the PCC 7120#11 microcosms had significantly higher PMO cell concentrations than the control microcosms, as indicated by *post hoc* tests. The high PMO cell concentration, approximately 6.8 log cells/ml, in the PCC 7120#11 microcosms decreased rapidly to approximately 6.4 log cells/ml by day 14 (Figure 5.6). After day 14, there was a slight but not significant increase in the PMO cell concentration in the PCC 7120#11 microcosms, followed by a "second" peak in the PMO cell concentration by day 21 (Figure 5.6). Thereafter, the PMO cell concentration declined gradually until day 35 (Figure 5.6). In the control microcosms, the PMO cell concentration increased until day 21, reaching a peak PMO cell concentration of approximately 6.5 log cells/ml (Figure 5.6). After day 21, PMO cell concentration decreased continuously until the end of the experiment (Figure 5.6). The trends in the log PMO cell concentrations were

similar in the control and PCC 7120#11 microcosms after day 14 (Figure 5.6). However, the main effect of treatment on total PMO cell concentration was statistically significant ($F_{1,10}$=6.040; p=0.0338), as was the interaction between time and treatment ($F_{10,100}$=7.230; p<0.0005). PCC 7120#11 microcosms had significantly higher PMO cell concentrations than the control microcosms on day 7, as shown by *post hoc* tests.

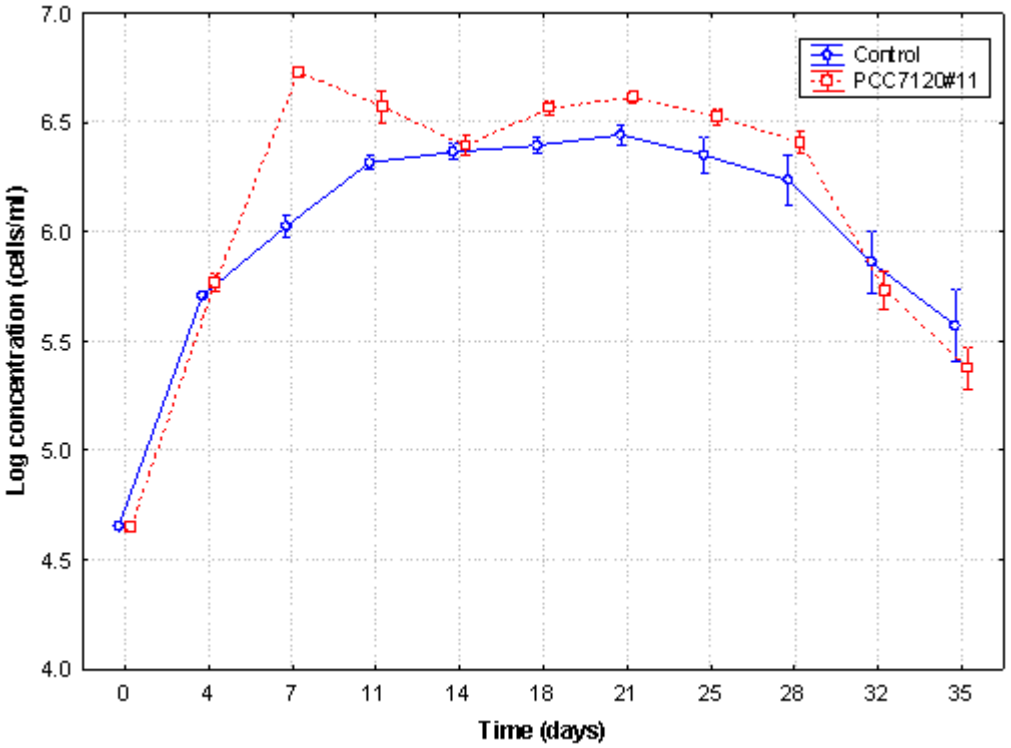

Figure 5.6 Change in the total photosynthetic microorganism concentration in control and PCC 7120#11 microcosms during the 35-day period. Values shown are mean value ± SE, n=6 for each data point.

Counts of the PCC 7120#11 cells showed that after an initial decrease of PCC 7120#11 between day 7 and day 14, the PCC 7120#11 cell concentrations plateaued at around 5.5 log cells/ml for 14 days (from day 14 to day 28), and then decreased to approximately 3 log cells/ml by day 35 (Figure 5.7).

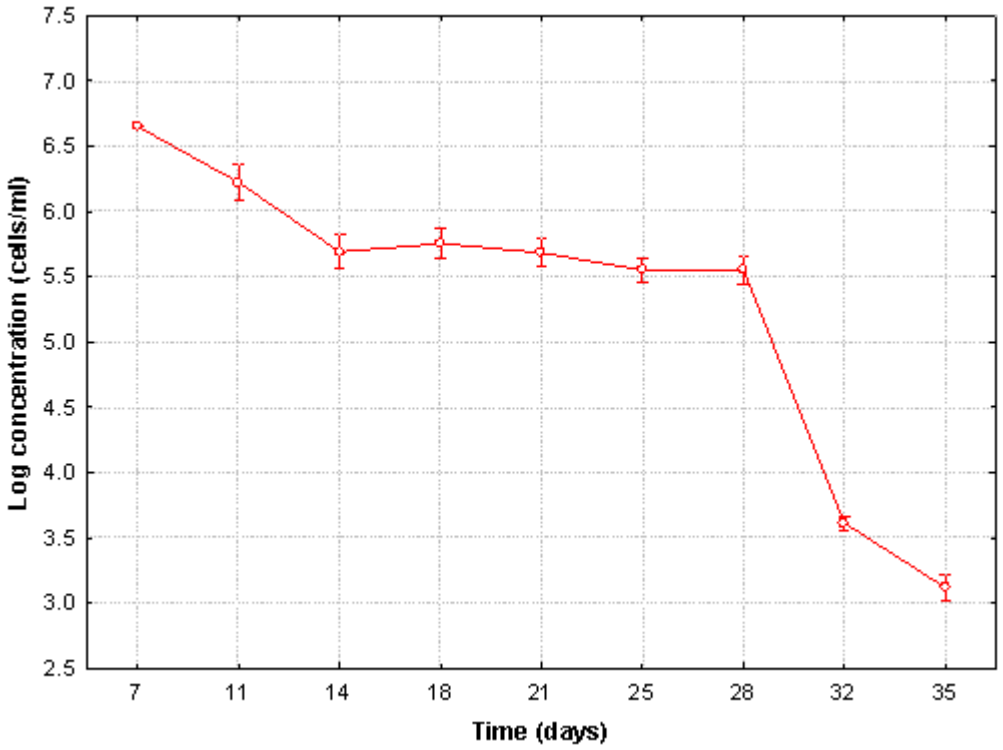

Figure 5.7 Change in PCC 7120#11 cell concentration in the PCC 7120#11 microcosms after the initial addition on day 7. Values shown are mean value ± SE, n=6 for each data point.

Figure 5.8 shows the change in concentration of the *D. pulex* populations throughout the 35 day period. The main effect of treatment on the concentration of *D. pulex* populations was not significant ($F_{1,10}$=2.197; p=0.1691), whereas the interaction between time and treatment was significant ($F_{9,90}$=2.644; p=0.0091), with *post hoc* tests indicating that the *D. pulex* population concentrations in the control microcosms were significantly higher on days 18 and 25 than in the PCC 7120#11 microcosms. However, the *D. pulex* population concentrations increased in a similar exponential-like manner in the control and PCC 7120#11 microcosms (Figure 5.8). Initially, *D. pulex* populations increased slowly in the control and PCC 7120#11 microcosms (Figure 5.8), but after day 11 the *D. pulex* populations in the control microcosms increased more rapidly than the *D. pulex* populations in the PCC 7120#11 microcosms (Figure 5.8). By day 32 both populations of *D. pulex* had peaked at the same concentration (Figure 5.8).

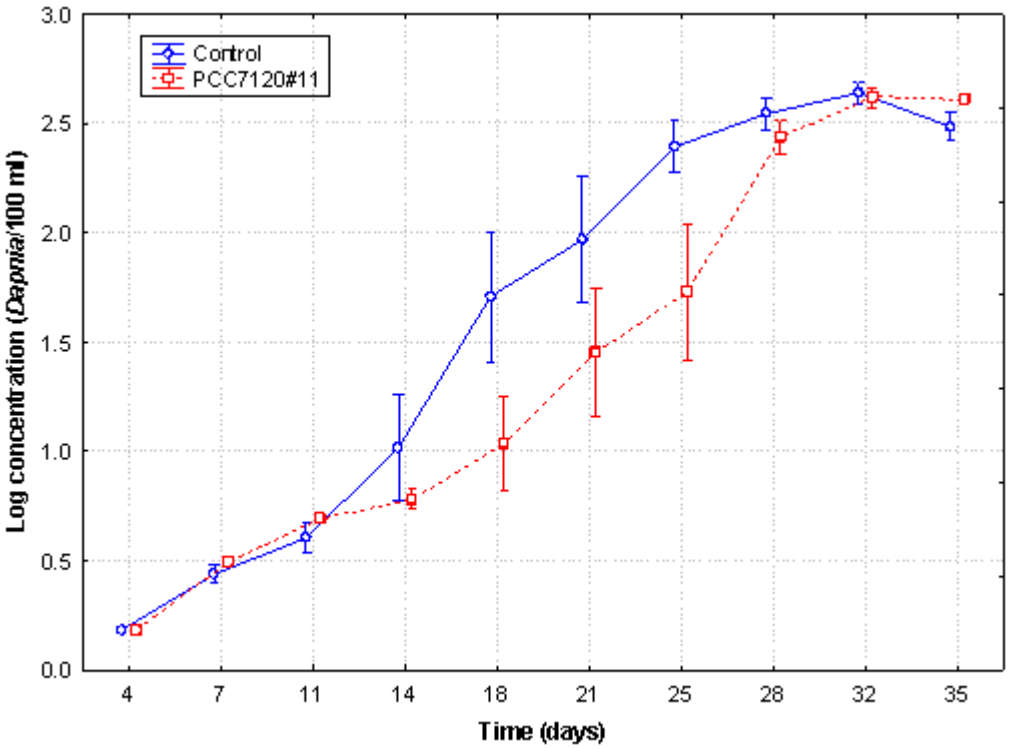

Figure 5.8 Change in *Daphnia pulex* population concentration in control and PCC 7120#11 microcosms during the 35-day period. Values shown are mean value ± SE, n=6 for each data point.

The changes over time in the concentration of *C. africanus* populations in the control and PCC 7120#11 microcosms were similar (Figure 5.9). The *C. africanus* populations exhibited a long lag phase from day 4 until day 11 (Figure 5.9). The changes in the *C. africanus* populations over time were cyclical whereby the populations would increase slightly and then plateau (Figure 5.9). After day 25, the *C. africanus* populations increased more rapidly in the control microcosms than in the PCC 7120#11 microcosms (Figure 5.9). *C. africanus* did not appear to flourish, as a maximum log concentration of approximately 0.60/100 ml ostracods was recorded in the control microcosms and a maximum log concentration of approximately 0.45/100 ml ostracods was observed in the PCC 7120#11 microcosms, by the end of the experiment. The main effect of treatment was statistically not significant ($F_{1,10}$=3.422; p=0.0941). Statistically, the interaction between time and treatment was significant ($F_{9,90}$=2.378; p=0.0184), with the *C. africanus* populations being

statistically higher in the control microcosms than in the PCC 7120#11 microcosms, on days 32 and 35 (*post hoc* tests).

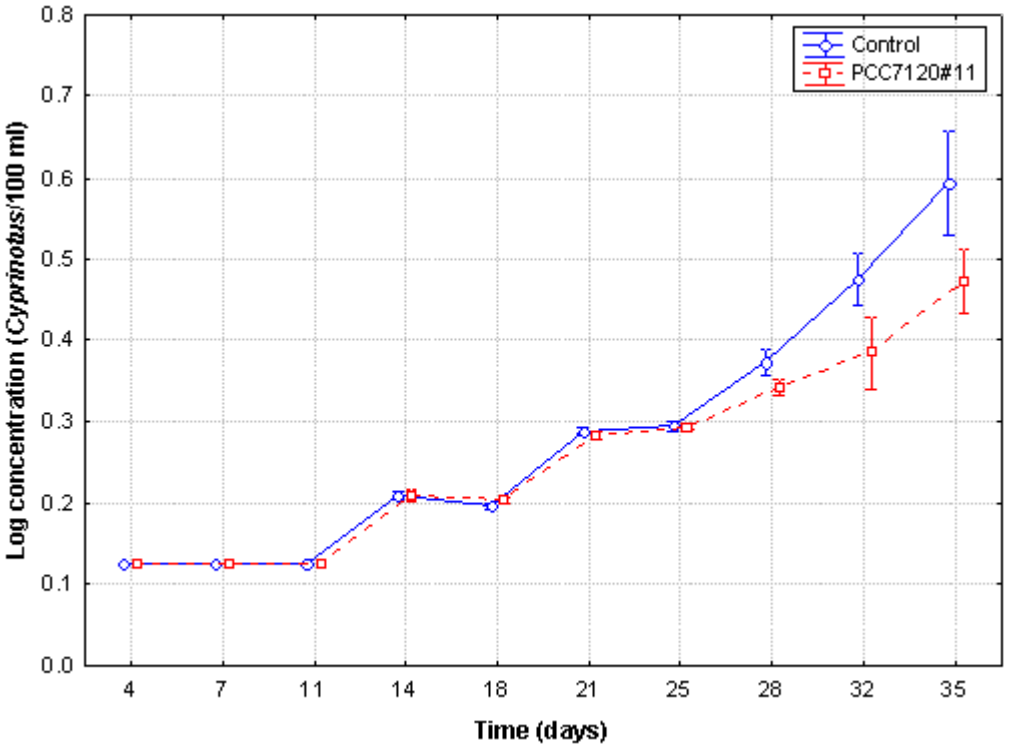

Figure 5.9 Change in *Cyprinotus africanus* population concentration in control and PCC 7120#11 microcosms during a 35-day period. Values shown are mean value ± SE, n=6 for each data point.

The *Lepadella* sp. population concentrations initially increased slowly (day 4 to day 14), then rapidly increased from day 14 until day 25 in the control and PCC 7120#11 microcosms (Figure 5.10). The main effect of treatment on the *Lepadella* sp. populations was not significant ($F_{1,10}$=4.466; p=0.0607), whereas the interaction between time and treatment, at day 32, was significant ($F_{9,90}$=2.319; p=0.0214) (*post hoc* tests). The *Lepadella* sp. population concentrations, on day 32, were significantly higher in the PCC 7120#11 microcosms than in the control microcosms. In the control microcosms peak concentrations were obtained by day 28 whereas in the PCC 7120#11 microcosms, peak concentrations were reached by day 32 (Figure 5.10).

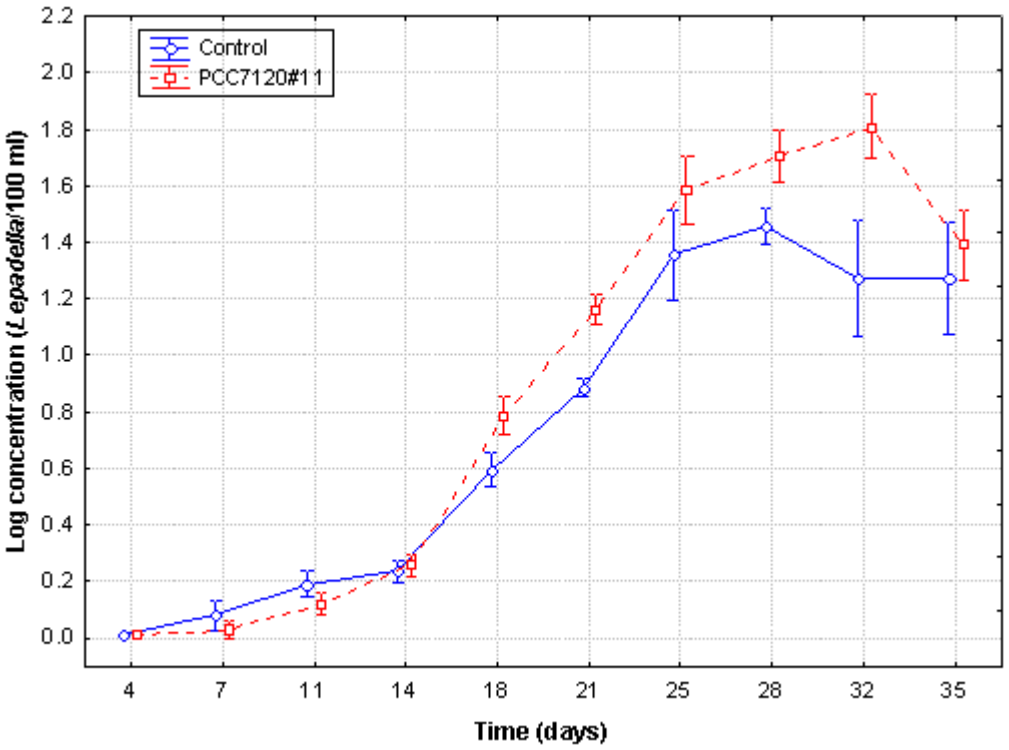

Figure 5.10 Change in *Lepadella* sp. population concentration in control and PCC 7120#11 microcosms during the 35-day period. Values shown are mean value ± SE, n=6 for each data point.

The changes in the *Coleps* sp. populations over time were similar in the control and PCC 7120#11 microcosms (Figure 5.11). The interaction between time and treatment was not significant ($F_{9,90}$=2.197; p=0.8614) and main effect of treatment was also not significant ($F_{1,10}$=4.550; p=0.0587). In both treatment groups, *Coleps* sp. populations increased rapidly from day 4 until day 14, then increased gradually to a maximum of approximately 2.3/100 ml (log value) protozoans by day 25 (Figure 5.11). Populations declined after day 25 (Figure 5.11).

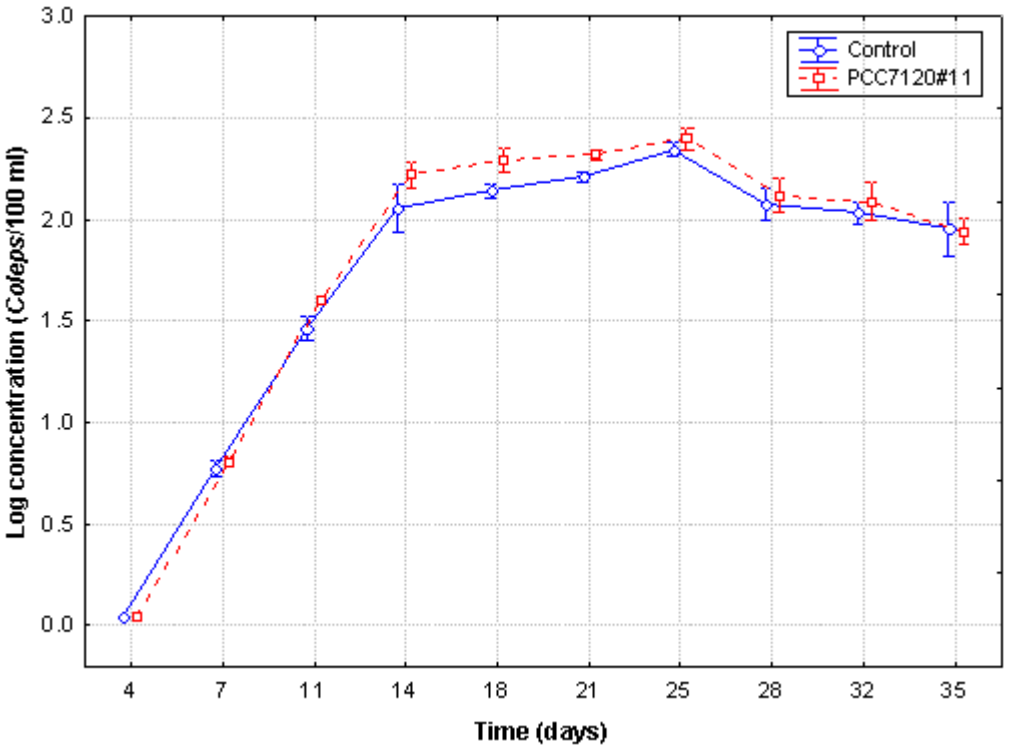

Figure 5.11 Change in *Coleps* sp. population concentration in control and PCC 7120#11 microcosms during the 35-day period. Values shown are mean value ± SE, n=6 for each data point.

5.5 Discussion

Laboratory bioassays have shown that some genetically engineered microorganisms have the potential to be efficient larvicidal agents (Yap *et al*., 1994a; Yap *et al*., 1994b; Liu *et al*., 1996; Armengol *et al*., 2005), however few studies have examined the larvicidal persistence and non-target effects of these agents in a comprehensive microcosm study. The genetically engineered cyanobacterium, PCC 7120#11 was introduced to six of the standardized microcosms on day 7. Twenty-four hours p.i. (day 8), the *An. arabiensis* larvae were reduced by 77%. The larvicidal activity of PCC 7120#11 did not persist in the microcosms, with the larvicidal activity being reduced substantially 7 days after the first inoculation (day 14). By day 14 the larvicidal activity was reduced to approximately 18% and from day 21 onwards there was no larval mortality in the PCC 7120#11 microcosms. The reduced larvicidal activity observed in this study could have been caused by several factors including a decrease in the concentration of PCC 7120#11,

either from reduced replication and/or cell death. The PCC 7120#11 cell concentration data showed that PCC 7120#11 cell concentrations initially decreased rapidly and then plateaued between day 14 and day 28. During the plateau phase the PCC 7120#11 cells may have been viable or may have been dead but still intact. The rapid decline in cell concentration after day 28 may reflect cell lysis of dead cells. Factors in the microcosm could possibly have limited the multiplication of PCC 7120#11, as previous studies have shown that PCC 7120#11 is capable of multiplying in mosquito breeding sites (Boussiba *et al*., 2000; Manasherob *et al*., 2002; Manasherob *et al*., 2003). In previous studies, it was reported (Chapter 3) that concentrations lower than 5.9 log cells/ml would result in reduced larvicidal activity against *An. arabiensis* larvae. Thus, a decrease in the concentration of PCC 7120#11 in the microcosms is likely to result in reduced larval mortality. Furthermore, to ensure that settling of the PCC 7120#11 cells did not limit the availability of the PCC 7120#11 cells to the larvae, prior to addition of the larvae, the microcosms were stirred.

Other environmental factors could have reduced the larvicidal activity of PCC 7120#11 in the microcosms, such as the amounts and composition of food present and the presence of other organisms competing for food. However, it is unlikely that the presence of other filter feeding organisms would have competed with the *An. arabiensis* larvae for the PCC 7120#11, as PCC 7120#11 is a filamentous cyanobacterium and the smaller, unicellular algae such as *C. vulgaris* and *S. capricornutum* are more favoured by the invertebrates used in this study (Thompson *et al*., 1982; Haney, 1987; De Mott *et al*., 1991).

The lack of persistence of larvicidal activity could have been caused by an increase in the availability of other food sources in the microcosms. In laboratory bioassays, the PCC 7120#11 cells were the only available food source for the mosquito larvae (Chapter 3). Thus, the larvae only ingest the

PCC 7120#11 cells, thereby resulting in high mortality. However, in the microcosms the presence and possible ingestion of other PMOs, protozoans, and bacteria by the mosquito larvae would reduce the intake of PCC 7120#11 cells (Walker *et al*., 1988; Thiery *et al*., 1991; Stevens *et al*., 1994; Marten, 2007), resulting in reduced to no mortality. Initially, on day 7, after the addition of PCC 7120#11, high larval mortality was observed possibly due to the fact that the other PMOs or protozoans were not readily available or were present at low concentrations, thereby resulting in more PCC 7120#11 being ingested. In addition to the observed decrease in PCC 7120#11 cell concentrations (after day 7), the increase in the total PMO cell concentrations and protozoan populations could have resulted in reduced *An. arabiensis* larval mortality.

The inorganic nutrients present in the control and PCC 7120#11 microcosms such as nitrate, nitrite, and phosphate had been depleted by day 18, as expected (Taub *et al*., 1988; Taub, 1989; Taub *et al*., 1991; Meador *et al*., 1993; Tsirtsis & Karydis, 1996). The increase in the total PMO cell concentration corresponded with a decrease in the above nutrients, as the PMOs utilize the inorganic nutrients for growth (Taub *et al*., 1988; Taub *et al*., 1991; Meador *et al*., 1993; Tsirtsis & Karydis, 1996; Taub, 2009). In the control and PCC 7120#11 microcosms, the concentration of ammonia was very low and only started to increase after day 28. Being a waste product, the level of ammonia increased as the number of invertebrates increased (Tsirtsis & Karydis, 1996). The presence of PCC 7120#11 did not appear to have a direct effect on the concentration of ammonia, as there was no significant difference in the concentrations of ammonia between the control and PCC 7120#11 microcosms.

In this study, there was a significant difference between the treatments when the nitrate and phosphate concentrations were examined. The interaction between time and treatment was also significant for both the nitrate and phosphate concentrations. The nitrate and phosphate concentrations were

significantly higher in the PCC 7120#11 microcosms on day 18 (for nitrate concentration) and on days 14 and 18 (for phosphate concentration). Previous studies evaluated the effect of the decomposition of algae on the levels of nitrate and phosphate (Jewell & McCarty, 1971; Lehman, 1980). Bacteria, as well as other microorganisms, can mediate nutrient cycling (De Pinto & Verhoff, 1977), whereby bacteria decompose the PMO cells releasing nitrate and phosphate that had been previously utilized by the PMOs (De Pinto & Verhoff, 1977). Bacteria were probably introduced in the microcosms with the addition of the other PMOs and invertebrates however, as per Taub (1989; 1997) the species and numbers were not monitored. Since bacteria are ubiquitous, it is possible that there were bacteria present in the microcosms that were capable of decomposition and nitrification of the dead PCC 7120#11 cells. Decomposition of PMOs is affected by several parameters such as light, temperature, nutrient content of the PMOs and media, and the microbial decomposer population (De Pinto & Verhoff, 1977). It has been shown that cyanobacterial cells lost cell structure rapidly during the decomposition process and that their decomposition is a rapid process, taking less than 16 days, provided the right parameters are present (De Pinto & Verhoff, 1977; Fallon & Brock, 1979).

In this study, after the addition of PCC 7120#11 (day 7), a decrease in the PCC 7120#11 cell concentration was apparent after 4 days probably due to the mortality of the PCC 7120#11 cells. Provided the conditions in the microcosms were favourable, including the presence of bacteria acclimated to decomposition and nitrification, the dead PCC 7120#11 cells could be rapidly decomposed resulting in higher phosphate and nitrate concentrations in the PCC 7120#11 microcosms than in the control microcosms. Phosphorus is released rapidly during the initial stages of the decomposition process (De Pinto & Verhoff, 1977), probably explaining the higher phosphate concentration observed in the PCC 7120#11 microcosms on day 14. The initial form of regenerated inorganic nitrogen is ammonia (De Pinto & Verhoff,

1977), which could account for the slight (but not statistically significant) peak in ammonia concentration observed, on day 14, in the PCC 7120#11 microcosms. The ammonia is then usually converted to nitrate by a bacterial nitrification process (De Pinto & Verhoff, 1977; Fallon & Brock, 1979), possibly explaining the higher nitrate concentration on day 18 in the PCC 7120#11 microcosms. The control microcosms did not have a high concentration of cyanobacterial cells added and hence decomposition was not likely to be so apparent. Future studies may want to examine the consortia of bacteria responsible for decomposition and also the rate at which decomposition is likely to occur.

Initially, the concentration of *D. pulex* populations was similar in the control and PCC 7120#11 microcosms. However, on days 18 and 25 the concentration of *D. pulex* populations in the PCC 7120#11 microcosms was significantly lower than those in the control microcosms. Although the interaction between treatment and time was significant on days 18 and 25, by day 32 the concentration of *D. pulex* populations in the PCC 7120#11 microcosms were very similar to the *D. pulex* population concentrations in the control microcosms. This suggests that PCC 7120#11 could have temporarily lowered *D. pulex* populations, with no long-term effects to the population concentrations (Taub *et al*., 1980). However, it is unlikely (but not proven) that *D. pulex* would ingest PCC 7120#11, as *Daphnia* sp. prefer the smaller, unicellular algae such as *C. vulgaris*, *S. capricornutum*, and *S. obliquus* (Thompson *et al*., 1982; Haney, 1987), suggesting that PCC 7120#11 may not have had a direct effect on the temporary lower *D. pulex* concentrations. The physical and chemical variables evaluated did not provide adequate reason for the temporarily lower *D. pulex* concentrations in the PCC 7120#11 microcosms. Turbidity, caused by organic (including PMOs), inorganic particles, and dissolved substances (Henley *et al*., 2000), has been shown to reduce the feeding efficiency of *D. pulex* (McCabe & O'Brien, 1983). However, both treatment groups had similar turbidity values (data not shown),

suggesting that turbidity was also not a factor in the lower *D. pulex* populations in the PCC 7120#11 microcosms.

The main effect of treatment on the *Coleps* sp. population concentrations was not significant, with the changes in the concentration of *Coleps* sp. populations in the PCC 7120#11 microcosms being similar to those in the control microcosms. The general trends of the *Coleps* sp. were different to those of *D. pulex*, *C. africanus*, and *Lepadella* sp. with the *Coleps* sp. increasing rapidly from day 4 and peaking before the other invertebrates. *Daphnia* sp. have been shown to be predators of protozoans (Pace & Vaqué, 1994), but there was no obvious reduction in *Coleps* sp. populations as the *D. pulex* concentrations increased. However, PCC 7120#11 did not affect the concentration of *Coleps* sp. populations in the microcosms, as there was no significant difference between the control and PCC 7120#11 microcosms.

The main effect of treatment on the *C. africanus* concentrations was not significant, but the interaction between treatment and time had a significant affect on the *C. africanus* concentrations. Although the *C. africanus* concentrations in the PCC 7120#11 microcosms followed similar trends to the control microcosms, *C. africanus* populations were significantly lower in the PCC 7120#11 microcosms than in the control microcosms on days 32 and 35. The significant difference observed towards the end of experiment is not necessarily a direct effect of PCC 7120#11, as the differences in the *C. africanus* population concentrations should have occurred much earlier, as has been shown in other studies where the treatment effect occurs within days of application (Taub *et al*., 1988; Taub *et al*., 1991; Sugiura, 1992; Meador *et al*., 1993). The concentrations of *C. africanus* populations in the control and PCC 7120#11 microcosms did not reach large numbers, remaining below 0.6 log ostracods/100 ml throughout the experiment. Thus slight variations in the reproduction rates of the *C. africanus* in the

microcosms could result in significant differences towards the end of the study (as indicated by the large standard error bars).

From day 18 onwards the *Lepadella* sp. population concentrations in the PCC 7120#11 microcosms were higher than those in the control microcosms, but the populations in the PCC 7120#11 microcosms were only significantly higher than those in the control microcosms on day 32. The concentration of *Lepadella* sp. populations declined after day 28 and day 32 in the control and PCC 7120#11 microcosms, respectively. This difference could be due to the fact that in the PCC 7120#11 microcosms the *D. pulex* populations were slightly lower than those in the controls. It has previously been reported that *Daphnia* sp. inhibit or kill rotifers by exploitative competition for shared, limited resources, and through mechanical interference (Gilbert, 1985; Conde-Porcuna, 1998). This suggests that the lower concentration of *D. pulex* populations in the PCC 7120#11 microcosms may not have reduced the concentration of the *Lepadella* sp. populations, thereby resulting in significantly higher populations of *Lepadella* sp. Importantly, the differences in the *Lepadella* sp. population concentrations should have occurred earlier in the experiment, rather than on day 32, as has been shown in other studies where the treatment effect occurs within days of application (Taub *et al*., 1988; Taub *et al*., 1991; Sugiura, 1992; Meador *et al*., 1993), thereby suggesting that PCC 7120#11 did not have an effect on the *Lepadella* sp.

There was no significant difference in net photosynthesis between the control and the PCC 7120#11 microcosms, indicating that the addition of PCC 7120#11 did not significantly affect photosynthesis. As the concentration of PMOs increased so did photosynthesis, similarly as the concentration of PMOs decreased so did photosynthesis, as has previously been found in other studies (Taub *et al*., 1991; Clément & Cadier, 1998).

PCC 7120#11 did not have a significant effect on the net photosynthesis, the ammonia and nitrite concentrations, or the *Coleps* sp. populations. The interaction between treatment and time on the *D. pulex*, *Lepadella* sp., and *C. africanus* populations was significant, however the main effect of treatment was not statistically significant. Importantly, by the end of the experiment there were no significant differences reported in the PCC 7120#11 microcosms for any of the above mentioned invertebrates, suggesting that PCC 7120#11 did not have a long-term effect on the invertebrate populations. This is important as it shows that although the PCC 7120#11 cells were prevalent at low concentrations in the microcosms for several weeks, PCC 7120#11 did not appear to have any long-term effect on the evaluated invertebrates and is thus unlikely to have an effect on non-target organisms in the field.

The main effect of treatment on the nitrate and phosphate concentrations was significant and *post hoc* tests showed that the interaction between treatment and time was significantly higher in the PCC 7120#11 microcosms than in the controls (on day 18 for nitrate and days 14 and 18 for phosphate). However, there was no significant difference in the nitrate and phosphate concentrations by the end of the experiment, indicating that the addition of a high concentration of PCC 7120#11 only temporarily increased the concentration of phosphate and nitrate in the microcosms. The slight increase in phosphate and nitrate concentrations did not appear to cause a spike in the total PMO concentration in the PCC 7120#11 microcosms. *Post hoc* tests showed that although there was a significant difference in total PMO concentration between the control and PCC 7120#11 microcosms after the addition of PCC 7120#11 (on day 7), by day 14 there was no significant difference in total PMO concentration between the control and PCC 7120#11 microcosms. PCC 7120#11 did not multiply in the microcosms and thus did not appear to out-compete the other PMOs. This is important for potential future applications of PCC 7120#11 to mosquito-breeding water bodies because PCC 7120#11 is unlikely to out-compete the natural PMO populations.

Although PCC 7120#11 exhibited larvicidal activity against the target organism, *An. arabiensis* larvae, the larvicidal activity was shown to decrease considerably from 77% to approximately 18% within 7 days. PCC 7120#11 persisted at low concentrations for several weeks in the microcosms, however the observed decrease in PCC 7120#11 cell concentrations (after day 7), in addition to the abundance of other smaller, unicellular algae and protozoa could have reduced the persistence of larvicidal activity of PCC 7120#11 against *An. arabiensis* larvae. Although there were temporary significant differences for some variables during the experiment, by the end of the experiment there were no significant differences between the control and PCC 7120#11 microcosms for any of the non-target organisms or other variables. This study was an important comprehensive study that showed that PCC 7120#11 is an environmentally safe agent, with no long-term effects on non-target organisms or water quality. PCC 7120#11 could play a role in integrated vector control programs.

Chapter 6

Evaluation of the optimal growing conditions of a recombinant cyanobacterium, *Anabaena* PCC 7120#11, in an indoor, flat-plate inclined photobioreactor using response surface methodology

6.1 Abstract

A recombinant *Anabaena* sp., *Anabaena* PCC 7120#11 has previously been shown to exhibit larvicidal activity against *Anopheles arabiensis*, a major vector in the transmission of malaria in Africa. The objective of this study was to investigate the optimal growth conditions of *Anabaena* PCC 7120#11 (PCC 7120#11), as PCC 7120#11 could have implications in control programs targeting malaria-carrying vectors. Response surface methodology, a collection of mathematical and statistical techniques, was used to design the experiments that would evaluate the combined effect of key growth factors (illuminance, air flow, and carbon dioxide) on the growth of PCC 7120#11, in an indoor, flat-plate inclined photobioreactor. The volumetric output rate (VOR) of the cultures was calculated and 3D surface response plots and a cube diagram were used to illustrate the effect and interactions of the three factors on VOR (g/l/d). The optimal conditions of growth were determined to be an illuminance of 5368 lux, with an air flow rate of 1.01 vvm and carbon dioxide input level of 3.74% (v/v). An optimal VOR of 0.48 g/l/d was obtained under these conditions. This study showed that the interaction of carbon dioxide and air flow played a significant role in the production of PCC 7120#11, as did the interaction of air flow and illuminance. The different combinations of the key growth factors evaluated did not significantly affect the larvicidal activity of PCC 7120#11 against *An. arabiensis*.

6.2 Introduction

Cyanobacteria are a widely distributed group of photoautotrophic, Gram-negative prokaryotes that have been used extensively for human and animal nutrition (Brown *et al*., 1999; Soletto *et al*., 2005; Spolaore *et al*., 2006), biofertilizers (Boussiba, 1991; Sinha & Häder, 1996), pharmaceuticals (Benemann *et al*., 1987; Belay *et al*., 1993), exopolysaccharides (Moreno *et al*., 1998), renewable energy, and biofuel production (Tsygankov *et al*., 2002; Yoon *et al*., 2002; Pulz & Gross, 2004; Yoon *et al*., 2006). More recently recombinant cyanobacteria have been shown to have a potential use as biopesticides against mosquitoes (Wu et *al*., 1997; Boussiba *et al*., 2000; Manasherob *et al*., 2002; Manasherob *et al*., 2003; Khasdan *et al*., 2003; Chapter 3).

Bacillus thuringiensis subspecies *israelensis* (*Bti*) is a Gram-positive, aerobic, spore-forming bacterium that produces crystalline inclusions that contain highly toxic crystal (Cry) and cytolytic (Cyt) proteins that have been shown to be toxic to the order Diptera (Goldberg & Margalit, 1977; Margalit & Dean, 1985; Feitelson *et al*., 1992; Crickmore *et al*., 1998; De Maagd *et al*., 2001). A filamentous, nitrogen-fixing cyanobacterium, *Anabaena* sp. strain PCC 7120, was genetically engineered to express the *Bti cry4Aa, cry11Aa*, and *p20* genes (Wu *et al*., 1997). The genetically engineered cyanobacterium, *Anabaena* PCC 7120#11, has been shown, in laboratory bioassays, to be a very effective larvicidal agent against *Aedes aegypti* (Wu *et al*., 1997; Lluisma *et al*., 2001; Manasherob *et al*., 2003) and several African *Anopheles* species (Chapter 3). The genus *Anopheles* plays a major role in the transmission of malaria in Africa, a disease causing over 1.2 million deaths a year (Gillies & De Meillon, 1968; Murray *et al*., 2012). Vector control is an important part of malaria prevention, with both the larval and adult stages of the vector being targeted in integrated vector control programs (Walker & Lynch, 2007; Beier *et al*., 2008). As a result of the high larvicidal activity of *Anabaena* PCC 7120#11 (hereafter referred to as PCC 7120#11) against several important

malaria vectors such as *Anopheles gambiae* Giles, *Anopheles arabiensis* Patton, and *Anopheles meras* Dönitz (Chapter 3), PCC 7120#11 could play a role in integrated vector control programs. Before PCC 7120#11 could be utilized in any vector control programs or commercialized, large-scale production of PCC 7120#11 would have to be evaluated and optimized.

Two major systems have been used for the large-scale production of cyanobacteria; the open cultivation system and the closed cultivation system (Posten, 2009). Open cultivation systems include outdoor shallow ponds (natural or artificial), raceway and circular ponds, and tanks (Richmond *et al*., 1990; Pulz, 2001). Although there are many advantages to using open cultivation systems such as easy scale-up, low operation, and investment costs, the main disadvantages of open cultivation systems are culture contamination risks and variable, uncontrollable environmental conditions such as maintaining optimal temperature, and cloudy, rainy days (Iqbal *et al*., 1993; Ugwu *et al*., 2002; Converti *et al*., 2006; Sierra *et al*., 2008). Closed systems, in particular indoor closed systems, have several advantages over open systems including better control of the important factors (such as temperature, illuminance, and carbon dioxide), reduced contamination risks, and more importantly reproducible cultivation conditions (Borowitzka, 1997; Borowitzka, 1999; Pulz, 2001).

There are several types of closed cultivation systems namely tubular, flat-plate, airlift, bubble-column photobioreactors, and stirred tank reactors (Richmond *et al*., 1993; Lee *et al*., 1995; Hu *et al*., 1996; Zhi & Rorrer, 1996; Tredici & Zitelli, 1998; Muller-Feuga *et al*., 1998; Zhang *et al*., 1999; Mirón *et al*., 1999; Ogbonna *et al*., 1999; Xu *et al*., 2002). The main factors that have to be considered if large-scale cultivation is to be successful include illuminance, carbon dioxide supply, nutrients, turbulence, temperature, and shear stress (Grobbelaar, 2000; Richmond, 2000; Pulz, 2001; Suh & Lee, 2003; Lehr & Posten, 2009). Illuminance is important because high illuminance can lead to

photoinhibition whereas low illuminance may limit photosynthetic activity and thus lower biomass productivity (Vonshak *et al*., 1982; Lee & Low, 1992; Richmond & Zou, 1999). The entire front surface area of the flat-plate, inclined photobioreactor is illuminated and flat-plate photobioreactors also have a very narrow light path, which ensures that the back of the photobioreactor also receives adequate illuminance (Hu *et al*., 1996; Richmond & Cheng-Wu, 2001). Turbulent streaming is also readily introduced in flat-plate photobioreactors and biofouling on the inner walls of the plate is more easily controlled (Iqbal *et al*., 1993; Richmond & Cheng-Wu, 2001). Thus, the main advantages that make flat-plate photobioreactors attractive for large-scale production of cyanobacteria include the high illuminance surface area, low accumulation of dissolved oxygen, low shear forces, high biomass concentrations, and convenience in scale-up (Iqbal *et al*., 1993; Cheng-Wu *et al*., 2001; Sierra *et al*., 2008; Xu *et al*., 2009). As a result of the combined advantages of flat-plate and closed indoor photobioreactors, a modified indoor, flat-plate inclined photobioreactor was evaluated in this study as a means to optimize the growth of PCC 7120#11.

Single factor optimization is commonly used in optimization studies whereby several factors are maintained at a fixed level, with successive changes in one independent variable (Elibol, 2004; Li *et al*., 2007). However, this method is time-consuming, costly, and requires a number of experiments to determine optimal levels (Elibol, 2004; Noordin *et al*., 2004; Duta *et al*., 2006). Not only are single factor optimizations tedious but they are often unreliable, especially as the interaction between different factors is often not evaluated (Abdel-Fattah *et al*., 2005; Bas & Boyaci, 2007; Li *et al*., 2007). To overcome the limitations of single factor optimizations, statistical designed experiments, which evaluate all the affecting factors collectively, have been used (Abdel-Fattah *et al*., 2005). One of the statistical designed experiments commonly used is the response surface methodology (RSM). RSM is a collection of mathematical and statistical techniques used for designing experiments and

models that can optimize the response by evaluating the relationships between the response and the independent variables to obtain desirable responses (Myers & Montgomery, 2002; Bas & Boyaci, 2007). In addition, it is a cost-effective and time-saving technique as it allows for the examination of many variables simultaneously (Noordin *et al*., 2004; Jacob-Lopes *et al*., 2008).

The objective of this study was to design and use an indoor, flat-plate inclined photobioreactor to determine the optimal growing conditions of PCC 7120#11, using the RSM approach. Factors such as turbulence, illuminance, and carbon dioxide concentration are important in the growth and development of cyanobacteria (Weissman *et al*., 1988; Thajuddin & Subramanian, 2005), hence this study investigated the effect that illuminance, air flow (turbulence), and carbon dioxide, have on the growth of PCC 7120#11. The larvicidal activity of PCC 7120#11 was evaluated against one of the main malaria vectors in southern Africa, *An. arabiensis*, to ensure that larvicidal activity was retained after PCC 7120#11 had been cultured in the photobioreactor, under the different culturing conditions evaluated.

6.3 Methodology

6.3.1 Culturing of PCC 7120#11

The genetically engineered cyanobacterium, PCC 7120#11 was cultured in BG-11 medium (Wu *et al*., 1997), at 30 °C under continuous illuminance (2000 lux) with constant agitation on a rotary shaker at 140 revolutions per minutes (Lluisma *et al*., 2001; Manasherob *et al*., 2003), until the mid-exponential growth phase. PCC 7120#11 was introduced into the flat-plate, inclined photobioreactor at an initial cell concentration of 1.6×10^6 cells/ml.

6.3.2 Flat-plate inclined photobioreactor design

A schematic diagram of the flat-plate inclined photobioreactor is given in Figure 6.1. The photobioreactor was set-up in an insulated walk-in incubator with the temperature set at 28 ± 2 °C.

The photobioreactor system consisted of two toughened glass plates (6 mm thick) measuring 702 mm long and 516 mm high. The light path length was 20 mm with a working volume of 3.7 litres (l) of the culture medium, BG-11. The glass plates were clamped onto the metal frame of the photobioreactor. Silicon tubing, which was affixed to the photobioreactor frame with marine sealant, prevented any leakage of culture. The photobioreactor was mounted at a 45° inclination on an adjustable stainless steel stand. The photobioreactor was continuously illuminated by a panel of eight Grolux (Sylvania, Germany) lamps that were placed above the photobioreactor. An illuminance meter, TES-1339 light meter (TES Electrical Electronic Corp., Taipei, Taiwan), was used to measure and monitor illuminance. The illuminance was modified by adjusting the distance between the light panel bank and the photobioreactor. Illuminance was evaluated between 500 to 7000 lux.

An air-bubble mixing system was used to mix the culture. The system consisted of a 0.5 cm diameter perforated tube running horizontally along the entire bottom length of the bioreactor. Air passed through the perforated tube (hole diameter 0.5 mm with 10 mm spaces between the holes) generating the required turbulence. Cultures were stirred continuously with compressed air enriched with carbon dioxide. The carbon dioxide/air mixture was filtered through a 0.2 µm (PTFE membrane) Pall Acro®50 Vent autoclavable air filter (Pall Corporation, New York, U.S.A.) before being introduced into the photobioreactor. The carbon dioxide/air mixture was controlled by adjusting the rate of gasses passing through three rotametres (Cole-Parmer, Chicago, U.S.A.) that measured the flow rates of the carbon dioxide, the air, and

mixture of the gases, respectively. The air flow was expressed in terms of litres of air per litre culture suspension per minute (vvm) (Hu *et al*., 1998a).

In order to maintain a constant volume, the reduction in liquid level caused by sampling was controlled by the addition of sterile distilled water. Losses in fluid due to evaporation were minimized by means of a 45-cm distillation condenser that was connected to a refrigerated chiller unit that maintained the condenser at 4 °C (Julabo F10, Julabo Labortechnik, Seelbach, Germany) (Figure 6.1). The temperature of the culture in the photobioreactor was monitored and maintained at a constant temperature of 28 °C. The photobioreactor was sterilized at the end of each run. The metal frame of the photobioreactor was rinsed with 10% (v/v) hypochlorite solution, scrubbed with 70% (v/v) ethanol, rinsed with distilled water and then autoclaved for 20 minutes. The glass plates were also washed twice with 10% (v/v) hypochlorite solution, rinsed with distilled water and then surface sterilized with 70% (v/v) ethanol.

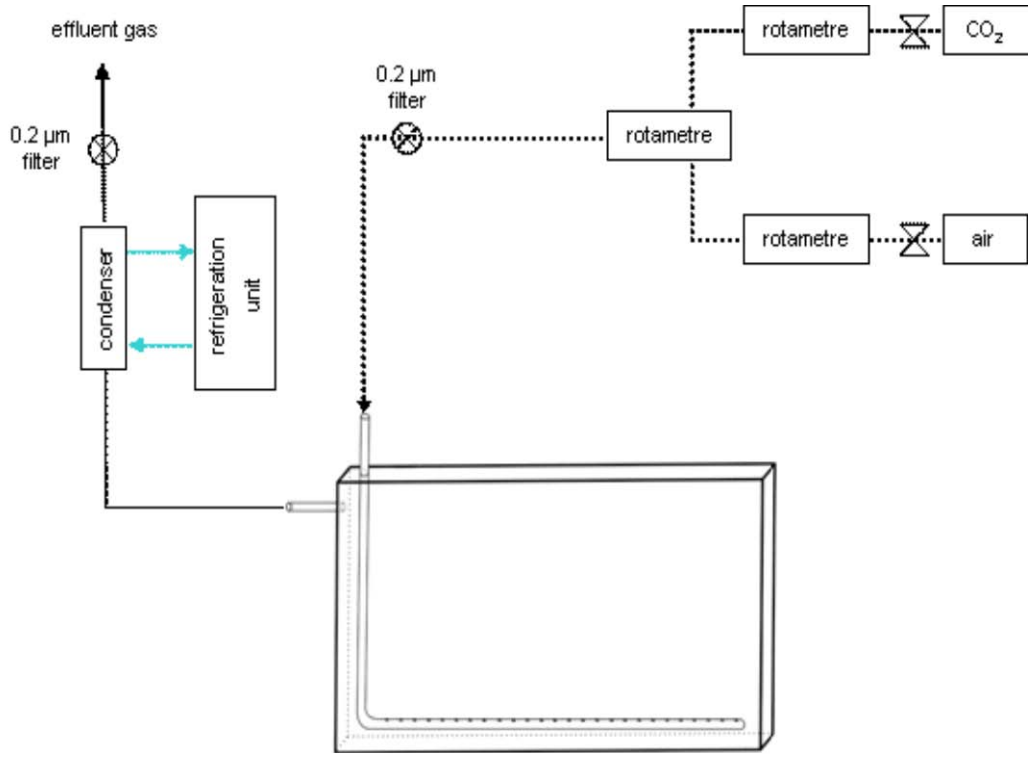

Figure 6.1 Schematic diagram of the flat-plate inclined photobioreactor (frontal view) and instrumentation.

6.3.3 RSM and statistical analysis

Design-Expert version 7.1.5 (Stat-Ease, Minneapolis, U.S.A.) was used to develop the experimental design and analyze the results. A RSM D-Optimal design (quadratic model) was used with numeric factors or variables (illuminance, air flow, and percentage of carbon dioxide). The experimental design is shown in Table 6.1. The upper and lower limits of illuminance, air flow, and percentage of carbon dioxide were determined in preliminary tests (data not shown). In order to verify the most appropriate model, several model diagnostic plots, such as the normal probability plot and the residual versus predicted plot, were performed. Box Cox plots were used to provide guidance on whether a power law transformation was required. Externally studentized residual plots were used to identify any outliers.

A test for the significance of individual model terms formed the basis for model optimization and model reduction was performed using backward elimination ($\alpha = 0.1$). The use of the backward elimination procedure reduces the model terms that are not significant, thus improving the model. Irrespective of their *p*-values, model terms required to support model hierarchy were not removed from the model during model reduction steps. During modelling, the adjusted R^2, the predicted R^2 and the precision of the model were also considered (Design Expert, version 7.1.5).

6.3.4 Determination of growth

Measurements of growth were performed every 12 hours. Dry weight of the cyanobacterial biomass was determined by filtering the cyanobacterial culture onto pre-weighed 1.2 µm pore-size glass microfiber filters (47 mm diameter) (Munktell & Filtrak, Bärenstein, Germany), followed by rinsing with sterile distilled water. The filters were dried at 80 °C for 24 hours and, after cooling to room temperature for 30 minutes in a desiccator, were weighed. Three measurements of biomass were performed for each sample period. The volumetric output rate (VOR) of the cultures was calculated and was reported as g/l/d.

Table 6.1 Experimental design for the optimization of *Anabaena* PCC 7120#11 growth using response surface methodology.

Run	Carbon dioxide (% v/v)	Air flow (vvm)	Illuminance (lux)
1	1.51	1.01	1818
2	2.63	1.75	3750
3	3.74	1.01	1818
4	3.74	2.49	1818
5	2.63	1.75	3750
6	2.63	1.75	3750
7	1.51	1.01	5682
8	3.74	1.01	5682
9	2.63	1.75	3750
10	1.51	2.49	5682
11	3.74	2.49	5682
12	1.51	2.49	1818
13	2.63	0.60	3750
14	2.63	1.75	3750
15	0.88	1.75	3750
16	2.63	3.00	3750
17	2.63	1.75	3750
18	2.63	1.75	500
19	4.5	1.75	3750
20	2.63	1.75	7000

6.3.5 Mosquito species

The anopheline mosquito species used was (origin and colony name provided) *An. arabiensis* (Kanyemba, Zimbabwe; KGB). The larvae were obtained from colonies maintained at the National Institute of Communicable Diseases in Johannesburg, South Africa. The larvae were reared in a controlled environment of 25 °C, relative humidity of 60%, and a L:D 12:12 hour photoperiod with a 45 minute dawn and dusk light regime.

6.3.6 Determination of larvicidal activity

For the laboratory bioassays, PCC 7120#11 cells were harvested at mid-exponential growth phase. The cells were harvested by differential centrifugation at 10 000 *g* (Beckman J2-21 with JA-14 rotor, Beckman Coulter, U.K.) for 25 minutes at 15 °C. The pellet was washed twice and then resuspended in a minimal volume of sterile distilled water and the cell concentration was determined by using a Neubauer haemocytometer (Superior Co., Berlin, Germany).

Ninety-eight millilitres of sterile distilled water was placed into 130 ml plastic cups to which twenty third instar mosquito larvae were added. Two millilitres of the appropriate dilution of PCC 7120#11 was added to the cups. Dilutions of the PCC 7120#11 were prepared in sterile distilled water to obtain the appropriate range of concentrations that would result in 5 to 95% larval mortality. The larvicidal activity was determined after 24 hours incubation in a controlled environment of 25 °C, relative humidity of 60%, and a L:D 12:12 hour photoperiod. Larvae were presumed dead if they did not move when prodded. An untreated control (2 ml of sterile distilled water) was included in the bioassays. If mortality in the controls exceeded 5%, the assay was discarded and repeated. The median lethal concentration (LC_{50}) of PCC 7120#11 against *An. arabiensis* larvae was determined by probit analysis (Finney, 1971). The probit analysis was based on the mortality data obtained from five PCC 7120#11 concentrations

6.4 Results

To evaluate the effect of the three key factors of growth on VOR, the runs were performed according to Table 6.1. The analysis of variance (ANOVA) for the response surface reduced quadratic model for the experimental design (Table 6.1) is shown in Table 6.2.

Table 6.2 Analysis of variance table (partial sum of squares) for Type III response surface reduced quadratic model for the response volumetric output rate (g/l/d).

Source	Sum of squares	d.f*	Mean square	*F*	*p>F*
Model	0.2851	6	0.0041	25.34	<0.0001
A-CO_2	0.0094	1	0.0475	4.999	0.0470
B-Air flow	0.0059	1	0.0094	3.140	0.1040
C-Illuminance	0.0949	1	0.0059	50.64	<0.0001
AB	0.0120	1	0.0949	6.436	0.0276
BC	0.0087	1	0.0121	4.657	0.0539
C^2	0.0539	1	0.0087	28.73	0.0002

***** degrees of freedom

The model for the effect of the three model terms on the VOR of PCC 7120#11 was significant (p<0.0001) (Table 6.2). The main effect of air flow (B) was not a statistically significant model term (p>0.1), whereas carbon dioxide (A) and illuminance (C) were significant model terms (Table 6.2). Although the main effect of air flow (B) was not a statistically significant model term (p=0.1040), it was added to the reduced model after the use of the backward elimination procedure to support hierarchy. Illuminance was the most significant model term affecting VOR (Table 6.2). The two-level interaction of carbon dioxide and air flow (AB), air flow and illuminance (BC), and the second-order effect of illuminance (C^2) were statistically significant model terms. The model terms A^2, B^2, and AC were removed as they were not significant (α=0.1).

The lack-of-fit was not significant (p>0.05), which is important because a significant value for the lack-of-fit would indicate that there are contributions in the regressor-response relationship that are not accounted for by the model (Noordin *et al*., 2004). In order to determine how well the model describes the experimental data, the various coefficients of determination (R^2) were evaluated. The R^2 value provides a measure of how much the variability in the observed response values can be explained by the model (Vohra *et al*.,

2002). The R^2 coefficients have values between 0 and 1 and the closer the value is to 1, the stronger the model (Li *et al*., 2007). For this model, the R^2 value was 0.933, which indicated that 93.3% of the variability in the response could be explained by the model. The adjusted R^2 compares models with different numbers of independent variables and corrects the R^2 value for the number of terms in the model (Vohra *et al*., 2002). The adjusted R^2 value (0.896) is in reasonable agreement with the predicted R^2 (0.685), which indicates a good agreement between the experimental and predicted values for VOR (Vohra *et al*., 2002). The adequate precision value compares the range of the predicted values at the design points to the average prediction error (Noordin *et al*., 2004). Ratios greater than 4 indicate that there is adequate model discrimination (Beg *et al*., 2002; Noordin *et al*., 2004). The adequate precision value of 16.5 obtained for our model suggests that there is adequate model discrimination.

The effect of the model terms carbon dioxide (A), air flow (B) and illuminance (C) on VOR can be explained by the following equation:

$$\mathbf{VOR\ (g/l/d) = -0.3857 + 0.0585\ (A) + 0.1810\ (B) + 0.0003\ (C) - 0.047\ (AB) - 2.30 \times 10^{-5}(BC) - 2.210 \times 10^{-8}\ (C^2)}$$

Three dimensional (3D) response surface plots, which are graphical representations of the regression equation, aid in the understanding of the interaction of the model terms and their optimal values required for optimization of VOR. The interactive effects of carbon dioxide and air flow, with a fixed illuminance (1818 lux), on VOR are exhibited in Figure 6.2. As the air flow increases from 1.01 to 2.49 vvm, there is a gradual decrease in VOR at a high carbon dioxide level (3.74% (v/v)) (Figure 6.2). However, as the carbon dioxide levels decrease from 3.74 to 1.51% (v/v), there is an increase in VOR at a high air flow of 2.49 vvm (Figure 6.2). The interaction of high carbon dioxide levels (3.74% (v/v)) and high air flow (2.49 vvm) or low carbon dioxide levels (1.51% (v/v)) and low air flow (1.01 vvm), resulted in sub-

optimal VOR (Figure 6.2). Under relatively low illuminance of 1818 lux, a VOR of approximately 0.27 g/l/d is achieved, when air flow is high and carbon dioxide level is low (Figure 6.2).

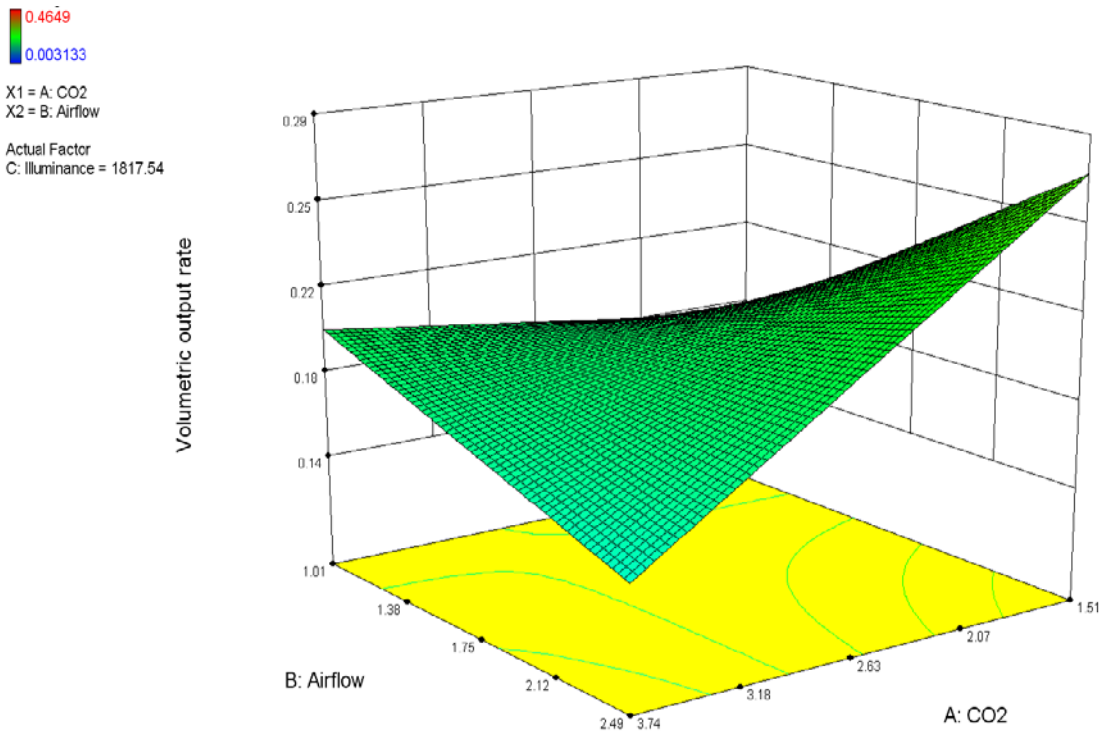

Figure 6.2 A 3D response surface plot exhibiting the effect of carbon dioxide (A) and air flow (B) on volumetric output rate (g/l/d) at an illuminance of 1818 lux. The colour key aids in interpreting the response surface plots by indicating where the optimal and sub-optimal VOR is expected to occur (the red colour and blue colour respectively on the key).

Figure 6.3 depicts how the VOR is affected by the interaction of air flow and carbon dioxide at a high illuminance value (5682 lux). At an illuminance of 5682 lux, the interaction between air flow and carbon dioxide is different to that observed in Figure 6.2. Under high illuminance, the maximum VOR (approximately 0.47 g/l/d) is achieved at high carbon dioxide levels (3.74% (v/v)) and low air flow (1.01 vvm). At a high carbon dioxide level (3.74% (v/v)), an increase in air flow from 1.01 to 2.49 vvm results in a reduction in VOR from approximately 0.47 to 0.20 g/l/d (Figure 6.3). Similarly at a high airflow (2.49 vvm), an increase in carbon dioxide levels from 1.51 to 3.74% (v/v)

results in a decline in VOR (approximately 0.42 to 0.20 g/l/d) (Figure 6.3). The lowest VOR (approximately 0.20 g/l/d) is obtained when the air flow rate and carbon dioxide level are at their highest (2.49 vvm and 3.74% (v/v), respectively). As indicated in Table 6.2 and in both Figures 6.2 and 6.3, the interaction of air flow and carbon dioxide had a significant effect on VOR.

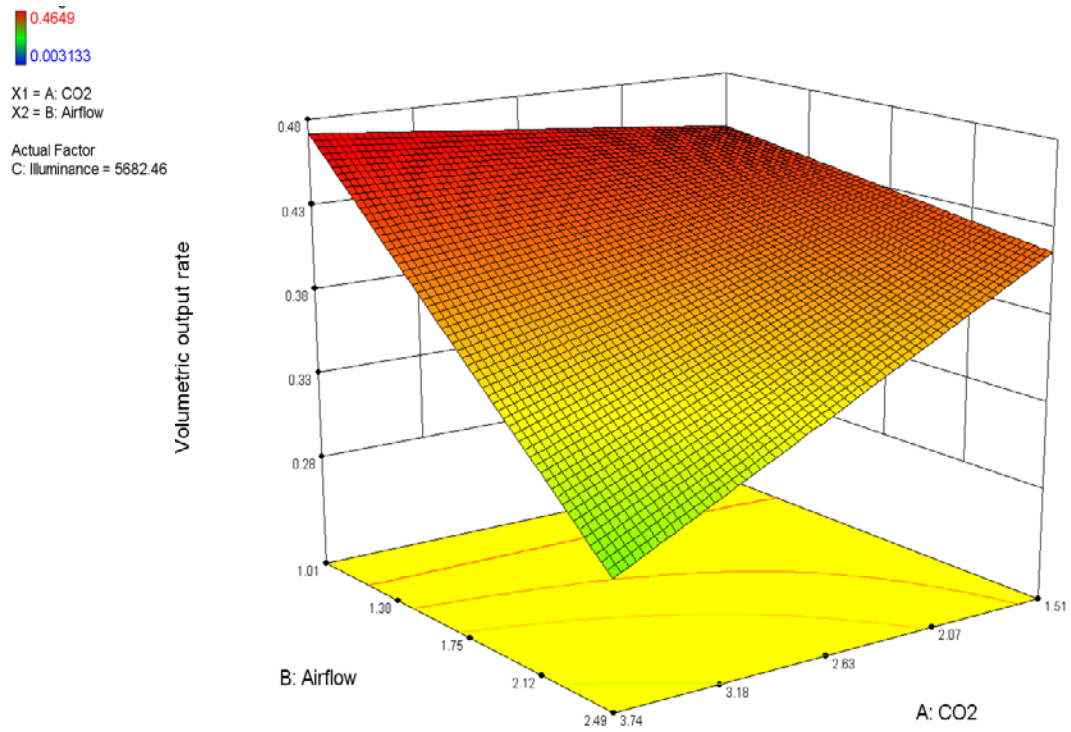

Figure 6.3 A 3D response surface plot exhibiting the effect of carbon dioxide (A) and air flow (B) on volumetric output rate (g/l/d) at an illuminance of 5682 lux. The colour key aids in interpreting the response surface plots by indicating where the optimal and sub-optimal VOR is expected to occur (the red colour and blue colour respectively on the key).

The interaction of the model terms, air flow and illuminance on VOR is shown in the 3D response surface plot in Figure 6.4, with the carbon dioxide level set at 2.63% (v/v). The maximum VOR (approximately 0.46 g/l/d), at a carbon dioxide level of 2.63% (v/v), is obtained when the air flow is 1.01 vvm and the illuminance is approximately 5600 lux. At low illuminance (1818 lux), the VOR is approximately 0.20 g/l/d and remains relatively unchanged as the air flow changes (Figure 6.4). At the highest illuminance (5682 lux), increasing the air

flow from 1.01 to 2.49 vvm decreased the VOR from approximately 0.46 to 0.35 g/l/d.

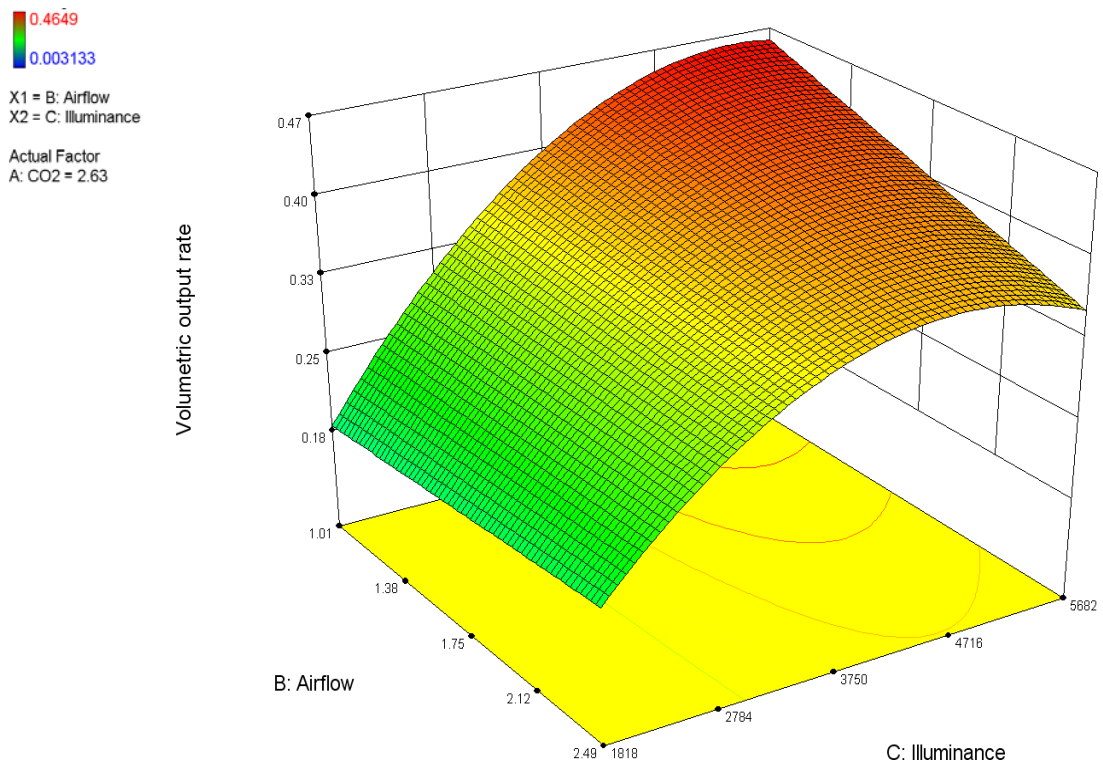

Figure 6.4 A 3D response surface plot exhibiting the effect of air flow (B) and illuminance (C) on volumetric output rate (g/l/d) at a carbon dioxide level of 2.63% (v/v). The colour key aids in interpreting the response surface plots by indicating where the optimal and sub-optimal VOR is expected to occur (the red colour and blue colour respectively on the key).

When the carbon dioxide level is set at 3.74% (v/v), a maximum VOR of 0.48 g/l/d is obtained at high illuminance (approximately 5600 lux) and low air flow (1.01 vvm) (Figure 6.5). Increasing air flow from 1.01 to 2.49 vvm at low illuminance (1818 lux), results in a change in the VOR from 0.22 to 0.12 g/l/d. Similarly at high illuminance (5682 lux), as the air flow increases from 1.01 to 2.49 vvm the VOR decreases from approximately 0.48 to 0.30 g/l/d (Figure 6.5). Figures 6.4 and 6.5 indicate the effect that the interaction of air flow and illuminance has on VOR, with higher carbon dioxide levels resulting in increased VOR.

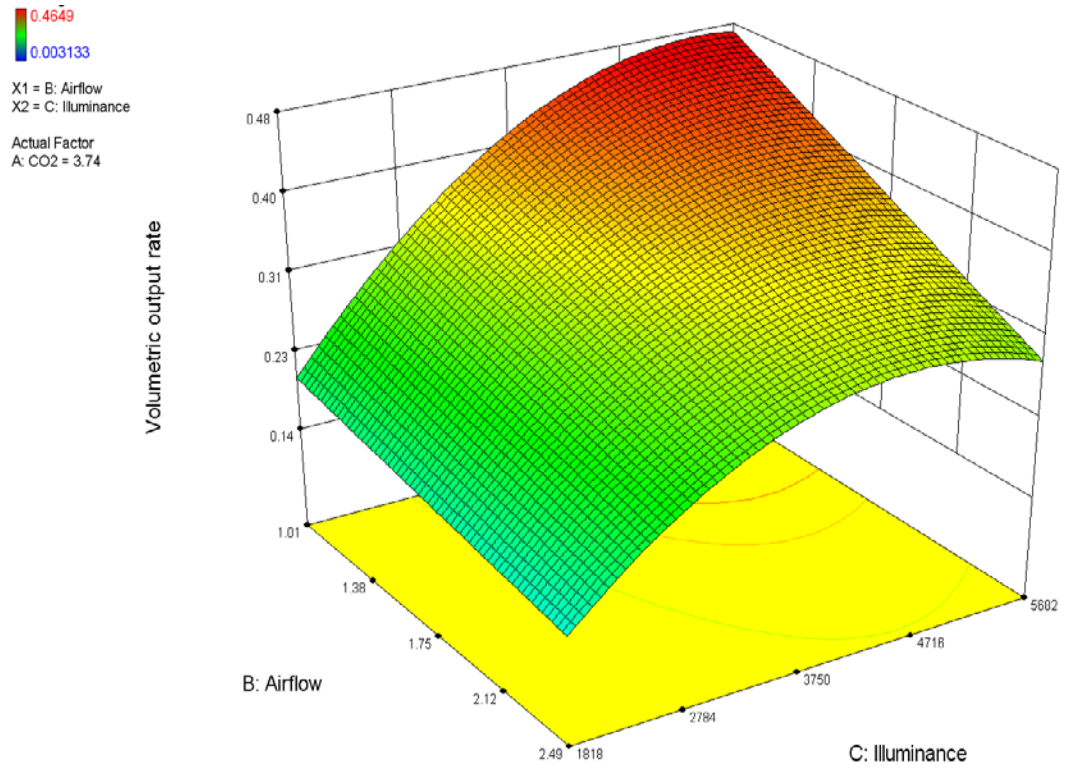

Figure 6.5 A 3D response surface plot exhibiting the effect of air flow (B) and illuminance (C) on volumetric output rate (g/l/d) at a carbon dioxide level of 3.74% (v/v). The colour key aids in interpreting the response surface plots by indicating where the optimal and sub-optimal VOR are expected to occur (the red colour and blue colour respectively on the key).

The interactions of only the maximum and minimum values of the three model terms on VOR is summarised in a cube diagram (Figure 6.6), with a high VOR (0.47 g/l/d) being obtained at the highest carbon dioxide (A+) and illuminance levels (C+) but at the lowest air flow rate (B-).

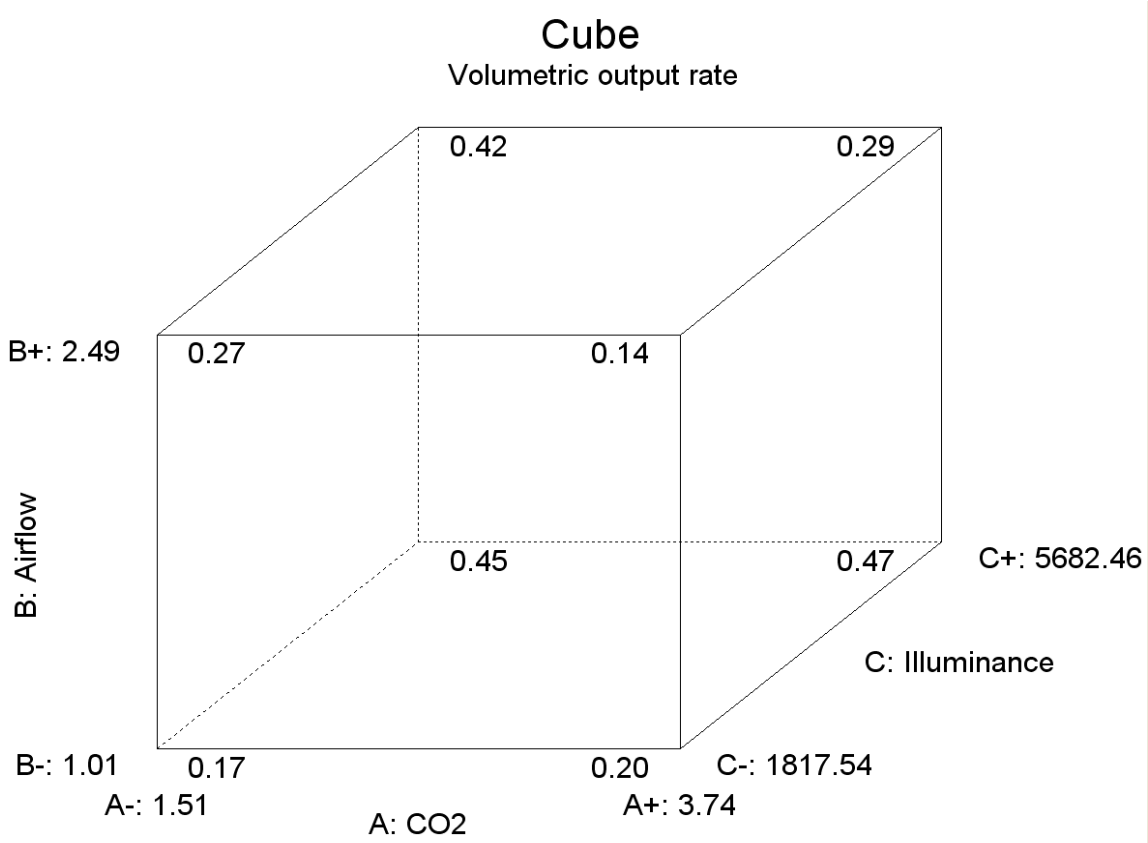

Figure 6.6 Cube plot showing the effect of the three model terms on volumetric output rate (g/l/d). The high and low values for carbon dioxide (A) are represented by A+ and A- respectively, and similarly the high and low values for air flow (B), and illuminance (C) are represented by B+ and B-, and C+ and C- respectively.

To evaluate if altering the levels of the key growth factors affected the larvicidal activity of PCC 7120#11, the LC_{50} for each experimental run was determined. At the mid-exponential growth phase of PCC 7120#11, an aliquot was removed and tested on third instar *An. arabiensis* larvae. A statistically significant RSM model could not be made to fit to the LC_{50} values (data not shown), suggesting that the model terms evaluated (illuminance, carbon dioxide, and air flow) did not significantly affect the larvicidal activity of PCC 7120#11.

The optimal conditions that lead to the optimal production of PCC 7120#11 in the flat-plate, inclined photobioreactor were determined to be an illuminance of 5368 lux, with an air flow rate of 1.01 vvm and carbon dioxide input level of 3.74% (v/v).

6.5 Discussion

Few studies have evaluated the interaction of the various factors of growth on cyanobacteria, in particular *Anabaena* sp. (Fontes *et al*., 1987; Yoon *et al*., 2008; Yu *et al*., 2011). The purpose of using RSM in this study was to aid in investigating the combination of the key growth factors that would result in the optimal production of PCC 7120#11. The cube diagram and 3D response plots were used to illustrate the effect and interaction of the three factors on VOR (g/l/d). This study demonstrated that there was a complex interaction between the evaluated factors of growth. For example, when all factors were high (illuminance of 5682 lux, air flow of 2.49 vvm, and carbon dioxide of 3.74% (v/v)), sub-optimal VOR was obtained. However, when only one of the factors, such as air flow was reduced to 1.01 vvm, an optimal VOR was achieved, and if only carbon dioxide was reduced (1.51% (v/v)), sub-optimal VOR was obtained.

Photosynthesis, which occurs in a photosynthetic unit (PSU), is described as the production and dissipation of an electrochemical gradient, whereby the oxidation of water provides a source of electrons (Falkowski & Kiefer, 1985; Cruz *et al*., 2005). The high-energy electrons are produced during photosynthesis when a non-activated PSU is activated by the absorption of photons (light) (Rubio *et al*., 2003). The electrons produced by the PSUs are utilized in enzyme-mediated reactions that result in the synthesis of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH), and the regeneration of PSUs (Rubio *et al*., 2003). ATP and NADPH reduce the available carbon dioxide to organic molecules in the Calvin cycle, ultimately leading to increased biomass (Papazi *et al*., 2008). Thus, the level of carbon dioxide introduced into the photobioreactor is important as it provides the only source of carbon for the PCC 7120#11 cells in the photobioreactor (Soletto *et al*., 2008; Xu *et al*., 2009; Dasgupta *et al*., 2010; Chiang *et al*., 2011). Research has shown that cultures (*Spirulina platensis*) grown at high illuminance levels (11100–17020 lux) are

characterized by higher production of electrons in their PSUs, which are indirectly used in the reduction of carbon dioxide (Soletto *et al*., 2008). Thus, high carbon dioxide levels and high illuminance should result in a high VOR based on the principle that at high illuminance there is an increased amount of electrons being produced. This would result in more activated PSUs (increasing photosynthesis), promoting the synthesis of more ATP and NADPH. As previously mentioned, ATP and NADPH reduce the available carbon dioxide to organic molecules, thus a high carbon dioxide level would result in high biomass or VOR. A high VOR (0.48 g/l/d) was observed in this study at the combination of high illuminance (5682 lux) and high carbon dioxide (3.74% (v/v)) levels however, RSM showed that optimal VOR is also dependent on the air flow rate (as will be discussed).

When the photons absorbed by the PSUs are too high, the concentration of electrons are in excess and they cannot be utilized by the Calvin cycle, resulting in damaged cell structure or photoinhibition (Demeter *et al*., 1995; Chojnacka & Noworyta, 2004). The range of illuminance (5682 to 1818 lux) evaluated in this study did not seem to promote photoinhibition but at the low end of the range, 1818 lux resulted in a lower VOR, suggesting that there was a limitation of energy available for photosynthesis (Jacob-Lopes *et al*., 2008).

In this study, the interaction of air flow and carbon dioxide was found to be a significant model term. This was expected as air flow aids in the distribution and uptake of carbon dioxide. At a high illuminance of 5682 lux, a high air flow rate of 2.49 vvm and a 3.74% (v/v) carbon dioxide level supplied the photobioreactor with a total volume of approximately 20.7 l/h of carbon dioxide. This resulted in a sub-optimal VOR of approximately 0.20 g/l/d. A higher VOR (approximately 0.42 g/l/d) was obtained when the total volume of carbon dioxide supplied was approximately 8.35 l/h (an air flow rate of 2.49 vvm and 1.51% (v/v) carbon dioxide). When the total volume of carbon dioxide supplied (at an air flow rate of 1.01 vvm and 3.74% (v/v) carbon

dioxide) was approximately 8.38 l/h, a VOR of approximately 0.47 g/l/d was obtained. This indicated that a very high total supply of carbon dioxide (20.7 l/h) did not result in optimal growth, whereas supplies of carbon dioxide below 9 l/h promoted optimal growth of PCC 7120#11. This suggests that the supply of total carbon dioxide, in combination with illuminance, is important for the optimal growth of PCC 7120#11. The total supply of carbon dioxide to the photobioreactor of approximately 8.4 l/h is comparable to previous studies that obtained optimal VORs at a total carbon dioxide supply of 9 l/h and 7.2 l/h (Zhang *et al*., 2001b; Zhang *et al*., 2002).

Previous reports have shown that increasing the gas flow rate decreased carbon dioxide fixation in the cells (Fan *et al*., 2007). This suggests that introducing carbon dioxide into the photobioreactor at a faster rate may reduce carbon dioxide uptake by the cells (decrease carbon dioxide fixation), at a particular illuminance, thereby influencing VOR. In this study, at an illuminance of 5682 lux, a low air flow rate (1.01 vvm) and carbon dioxide level of 3.74%, VOR was higher than at high air flow (2.49 vvm) and high carbon dioxide (3.74% (v/v)) level. An optimal air flow rate of 1.01 vvm at high carbon dioxide levels (3.74% (v/v)) (at an illuminance of 5682 lux) could have lead to optimal VOR because the carbon dioxide bubble retention time was prolonged, allowing the cyanobacterial cells to take up the carbon dioxide more sufficiently (Fan *et al*., 2007).

The interaction of air flow and illuminance was also found to be a significant model term. Previous studies have shown that in most photobioreactors, air flow carries the cells through both the well-illuminated and poorly illuminated areas in the photobioreactor ensuring that the majority of the cells are exposed to the light (Fontes *et al*., 1987; Hu *et al*., 1998a; Hu *et al*., 1998b). The design of the flat-plate photobioreactor utilized in this study (a short path length and illumination of the entire front surface of the photobioreactor) ensured that there were no poorly illuminated areas. However, at high cell

densities the cells shade each other (mutual cell shading), known as the light shading effect, from the illuminance, thus air flow is important to ensure that there is adequate mixing to prevent shading of the PCC 7120#11 cells (Grobbelaar *et al*., 1995; Ugwu *et al*., 2008). Air flow is also required to prevent the PCC 7120#11 cells from settling, and thus ensure exposure to the illumination supplied. More importantly, as previously mentioned, air flow ensures the delivery of carbon dioxide to the cyanobacterial cells which is then utilized for photosynthesis by the PCC 7120#11 cells (provided the illuminance is adequate) to increase biomass and hence VOR.

The larvicidal activity of PCC 7120#11 was investigated to ensure that the variations in the key growth factors did not affect the production of the Cry proteins. The LC_{50} values (data not shown) obtained against the target mosquito species, *An. arabiensis*, at each run were within the range of the LC_{50} value of 8.10 x 10^5 cells/ml obtained in a previous study (Chapter 3), with some values being lower (4.0 x 10^5 cells/ml) and others being higher (9 x 10^5 cells/ml).

The optimal volumetric output rate of PCC 7120#11 in this study was 0.48 g/l/d with a culture volume of 3.7 l, and air flow rate of 1.01 vvm at 5368 lux. Due to differences in photobioreactor design and experimental conditions, such as different types of lighting used, different air flow rates, different carbon dioxide levels, care should be taken when comparing different studies. In addition to the above differences, different species of cyanobacteria show marked differences in their response to carbon dioxide levels and illuminance (Yoon *et al*., 2002). Our results are comparable to the range of VORs determined by other research studies. It has been shown that *Anabaena variabilis* cultured in a bubble column reactor, with an illuminance of 7400-8140 lux, had a VOR of 0.98 g/l/d (Yoon *et al*., 2008) and the VOR of *Spirulina* sp. in an indoor, 4.8 l flat chamber reactor was 1.93 g/l/d at an air flow rate of 1.65 vvm (Tredici & Zittelli, 1998). A recent study by Yu *et al*.

(2011) on the growth of *Anabaena* sp. strain PCC 7120 (wild type) in a bubble column reactor, supplied with glucose and 3% carbon dioxide, reported a VOR of approximately 0.32 g/l/d. This suggests that *Anabaena* sp. strain PCC 7120 may have a slower growth rate than the other cyanobacteria previously evaluated, suggesting that a lower VOR may be expected for PCC 7120#11. A further point to consider is that PCC 7120#11 is a recombinant cyanobacterium that has been genetically modified to produce Cry proteins. This may result in a lower VOR observed for PCC 7120#11 when compared to the other cyanobacteria, as PCC 7120#11 may respond differently to key growth factors than other cyanobacteria.

This study showed that the interaction of carbon dioxide and air flow played a significant role in the optimal production of PCC 7120#11, as did the interaction of air flow and illuminance. This study showed that a high illuminance increased the photosynthetic activity of the PCC 7120#11 cells, however it has to be supported by a suitable carbon dioxide feeding rate (interaction between carbon dioxide level and air flow rate). The conditions that lead to the optimal growth of PCC 7120#11 in the flat-plate, inclined photobioreactor were determined to be an illuminance of 5368 lux, with an air flow rate of 1.01 vvm and carbon dioxide level of 3.74% (v/v). The running costs of photoautotrophic production systems are always important, with the cost of carbon dioxide playing a role in determining the cost-effectiveness of the photobioreactor (Cheng-Wu *et al*., 2001). In this study, RSM showed that although a high total carbon dioxide supply (8.38 l/h) was optimal for maximum VOR (0.48 g/l/d) (under the combinations evaluated), a slightly lower carbon dioxide supply (2.63%) at the same air flow rate (1.01 vvm) would still yield a high VOR (0.46 g/l/d), thus reducing the running costs of the photobioreactor. RSM proved to be a very good approach in determining the complex interactions of the three key growth factors of PCC 7120#11 that are necessary for the optimal growth of PCC 7120#11. Furthermore, this study showed that different factor level combinations of the key growth factors did

not significantly affect the larvicidal activity of PCC 7120#11 against *An. arabiensis*. This study showed that PCC 7120#11 can be produced in a flat-plate, inclined photobioreactor and can thus be used in future scale-up studies, as a precursor to potential commercialization of PCC 7120#11.

Chapter 7

General discussion and conclusion

7.1 General discussion

Malaria, one of the most important parasitic diseases in the world, is a vector-borne disease caused by protozoan parasites of the genus *Plasmodium* (Cowman & Crabb, 2006). Malaria is transmitted by the bite of an infected female *Anopheles* mosquito (Cowman & Crabb, 2006). Effective control strategies for malaria vectors pose a great challenge, as no single strategy is applicable for all situations (Rose, 2001; Takken & Knoll, 2008). Chemical insecticides have been used in control programs; however, the development of resistance and the negative impact of chemical insecticides on the environment have promoted the search for alternative control strategies (Ranson *et al*., 2001; Rose, 2001; Hougard *et al*., 2002; Federici *et al*., 2003; Coetzee, 2004; Takken & Knols, 2008).

The use of biological control agents, in particular *Bacillus thuringiensis* subspecies *israelensis* (*Bti*) based biopesticides, provides an environmentally friendly strategy for the control of malaria vectors (Lacey *et al*., 2001; Federici *et al*., 2003; Crickmore, 2006). A recombinant *Anabaena* sp. strain PCC 7120, *Anabaena* PCC 7120#11, that carries specific *Bti* toxin genes was shown to be an effective larvicidal agent in laboratory assays (Wu *et al*., 1997; Lluisma *et al*., 2001; Manasherob *et al*., 2003). This suggested that it may have potential as a control agent of malaria mosquito vectors in southern Africa. Thus, the main objective of this thesis was to evaluate *Anabaena* PCC 7120#11 (hereafter referred to as PCC 7120#11) as an agent to control southern African malaria vectors. PCC 7120#11 was evaluated as an alternative mosquito biological control agent to *Bti* (the standard biological control agent), as *Bti* has several shortcomings as a biological control agent (Ohana *et al.*, 1987; Sheeran & Fisher, 1992; Manasherob *et al*., 2003).

Several specific aims were evaluated in order to determine the potential of PCC 7120#11 to control southern African malaria vectors. The larvicidal activity of *Bti* against several *Anopheles* species was evaluated in Chapter 2

as no studies, to our knowledge, have evaluated comprehensively the larvicidal activity of *Bti* on all the important African anopheline vectors. The larvicidal activity of PCC 7120#11 against several *Anopheles* species was addressed in Chapter 3. Chapter 4 evaluated the effects of *Bti* on non-target organisms in a standardized aquatic microcosm (SAM) and was the basis for comparison to the PCC 7120#11 microcosm study (Chapter 5), whereby the potential environmental risks of PCC 7120#11 were evaluated. Finally, Chapter 6 determined the optimal growth of PCC 7120#11, as a precursor to potential commercialization.

The first aim of this thesis (Chapter 2) was to determine the susceptibility of five anopheline species to *Bti*. If the anopheline species were susceptible to *Bti*, then they are likely to be susceptible to other *Bti*-based biopesticides such as PCC 7120#11, thereby allowing for comparison of the relative susceptibilities of *Bti* and PCC 7120#11. Any differences in susceptibilities between PCC 7120#11 and *Bti* could then be addressed. As no studies, to our knowledge, have comprehensively evaluated the larvicidal activity of *Bti* on all the important African anopheline vectors, the larvicidal activity of *Bti* against each anopheline species in Chapter 2 provided a point of reference for comparison against PCC 7120#11.

This study showed that all five of the anophelines evaluated (*An. gambiae*, *An. arabiensis*, *An. funestus*, *An. quadriannulatus*, and *An. merus*) were susceptible to *Bti* with *An. quadriannulatus* being statistically the most susceptible to *Bti*, with an LC_{50} value of 2.97×10^4 spores/ml. Although there was no significant difference in susceptibility, at the LC_{50}, between the other vectors evaluated, *An. funestus* had the lowest LC_{50} value of 4.4×10^4 spores/ml. The LC_{50} values obtained in our study are within the ranges published for other anopheline larvae (Panbangred *et al*., 1979; Lacey & Singer, 1982; Wraight *et al*., 1987). Evaluating the susceptibility of important malaria vectors to *Bti* was achieved in Chapter 2, indicating that there is a

potential to control each of the anopheline species evaluated, however further evaluation is required.

Based on the susceptibility of the five anopheline species to *Bti*, the larvicidal activity of PCC 7120#11 against the same five anopheline species (Chapter 3) was expected to result in a similar susceptibility ranking to that obtained for *Bti*. However, the study showed that the susceptibility ranking of the five anopheline species to PCC 7120#11 was not similar to that obtained for *Bti*. *An. quadriannulatus* was statistically the least susceptible to PCC 7120#11, whereas it was statistically the most susceptible to *Bti* (Chapter 2). Amongst the anopheline species, PCC 7120#11 exhibited the highest larvicidal activity against *An. merus* with an LC_{50} value of 3.90×10^5 cells/ml. Fifty percent mortality was not obtained even at concentrations as high as 3.20×10^7 cells/ml for *An. funestus*, suggesting that PCC 7120#11 was not larvicidal to *An. funestus* at feasible concentrations. PCC 7120#11 was shown to be larvicidal against *An. gambiae* and *An. arabiensis* at LC_{50} values of 12.3×10^5 cells/ml and 8.10×10^5 cells/ml, respectively.

The difference in relative susceptibilities or larvicidal ranking between the anopheline species to *Bti* and PCC 7120#11 suggests that the species were less susceptible to the combination of the toxins (Cry4Aa and Cry11Aa proteins) in PCC 7120#11 than to *Bti*, which naturally produces the toxins Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa and Cyt2Ba (Höfte & Whiteley, 1989; Ben-Dov *et al*., 1999; Berry *et al*., 2002). It has been shown that *Bti* has higher larvicidal activity against mosquitoes than recombinants containing a subset of *Bti* toxins (Delécluse *et al*., 1993; Poncet *et al*., 1995; Schnepf *et al*., 1998). The absence of certain toxins (Cry4Ba, Cry10Aa, Cyt1Aa, and Cyt2Ba) in PCC 7120#11 may have resulted in the low larvicidal activity of PCC 7120#11 against *An. funestus* larvae, and the different combinations of *Bti* toxins in PCC 7120#11 and *Bti* may have resulted in differences in the

susceptibility ranking of the anopheline species between PCC 7120#11 and *Bti*.

The differences observed in susceptibility between the anopheline species to PCC 7120#11 and *Bti* could also be due to differences in the structure and/or density of specific midgut toxin receptors for the Cry4Aa or Cry11Aa proteins, as previous studies have shown that the larvicidal activity of *Bti* Cry toxins is often correlated to the binding of the toxin to the specific membrane-bound receptors in the larval midgut (Hofmann *et al*., 1988; Van Rie *et al*., 1990; Ravoahangimalala *et al*., 1993; Ravoahangimalala & Charles, 1995). Inherent physiological differences in the midguts of the anopheline species evaluated, in particular between *An. funestus* in the *An. funestus* group and members of the *An. gambiae* complex, may affect the binding of the toxins. However, this hypothesis would need to be confirmed by detailed studies of the binding of the different Cry proteins to the midgut receptors of the different species evaluated.

The differences observed in susceptibility between the *Anopheles* larval species evaluated could also be partly attributed to the differences in feeding behaviour and feeding rate between the species (Tyrell *et al*., 1979; Lacey & Singer, 1982). Studies on the feeding behaviour of the five anopheline species evaluated may clarify if the differences in susceptibilities to *Bti* and PCC 7120#11 could be attributed to differences in feeding rate and behaviour.

The concentration-mortality regression slopes obtained in Chapters 2 and 3 indicate the variability in response to the toxin within the vector population being examined. The regression slopes obtained in Chapter 2 (*Bti* bioassays) for *An. quadriannulatus* and *An. merus* were significantly steeper than those of all the other anopheline species examined, thereby indicating that *An. quadriannulatus* and *An. merus* had lower population response variability to *Bti* than the other mosquito populations evaluated. The relatively shallow

slopes of the concentration-mortality regression lines obtained for some of the anopheline species evaluated means that there are larger differences between the LC_{50} and LC_{90} values for these species than for the other species. In Chapter 3 (the PCC 7120#11 bioassays), *An. gambiae* and *An. quadriannulatus* had larger differences between the LC_{50} and LC_{90} values than the other species evaluated (based on their statistically shallower slopes of the concentration-mortality regression lines). *An. arabiensis* had a significantly steeper slope than the other species evaluated, suggesting that *An. arabiensis* had the lowest population response variability. Since LC_{90} values, rather than LC_{50} values form the basis of control program application rates, it may be worthwhile to evaluate several different populations of vectors to ascertain if the heterogeneity is species specific rather than population specific.

Chapter 3 showed that third instar *A. aegypti* larvae, included as a control in the bioassays because the activity of PCC 7120#11 against this mosquito species had been previously reported by Wu *et al*., (1997), had an LC_{50} value of 1.42×10^5 cells/ml. This was comparable to the LC_{50} value of 0.9×10^5 cells/ml reported by Wu *et al*. (1997). The larvicidal activity of PCC 7120#11 against several of the anopheline species evaluated suggests that PCC 7120#11 has the potential to be used as a larvicidal agent as part of integrated vector control programs.

However, without evaluating some of the potential risks associated with releasing a genetically engineered cyanobacterium into the environment, it is unlikely that any regulatory body would approve the release of PCC 7120#11 in the field. The evaluation of non-target effects of PCC 7120#11 was the focus of Chapter 5. As a secondary focus, Chapter 5 also examined the persistence of the larvicidal activity of PCC 7120#11 and the persistence of the PCC 7120#11 cells in the microcosms. Ideally, PCC 7120#11 should not persist for a long period of time in the environment, as this would pose a

problem with regards to obtaining regulatory approval. The use of the SAM protocol provided valuable information on the effects of *Bti* (Chapter 4) and PCC 7120#11 (Chapter 5) on several variables, including non-target organisms and chemical and physical variables.

Considering that numerous studies have different experimental designs and methodologies which could lead to different conclusions with regards to the effect of *Bti* on non-target species and persistence (Boisvert & Boisvert, 2000; De Melo-Santos *et al*., 2009), and since *Bti* is the standard biopesticide currently used for mosquito control (Lacey *et al*., 2001; Crickmore, 2006), the aim of Chapter 4 was to use the more sensitive and reproducible SAM protocol to provide a comprehensive evaluation of the effects of *Bti* on several variables. The SAM experiment would thus eliminate many uncertainties, with regards to the effect of *Bti* on non-target organisms that may currently exist. The SAM protocol was also used to assist in determining if PCC 7120#11 may be a more environmentally friendly control agent than the more widely used *Bti*. Thus, Chapter 4 provided the basis for comparison of PCC 7120#11 to *Bti*. Although several anopheline species were evaluated in this thesis, *An. arabiensis* was selected as the target organism in the microcosm studies as it is the most important malaria vector in southern Africa (Coetzee *et al*., 2000), and was susceptible to PCC 7120#11 and *Bti*.

Multiple additions of *Bti* were applied to the microcosms as *Bti* is not expected to maintain its larvicidal activity for more than 7 days in an aquatic environment (Mulligan *et al.*, 1980; Davidson *et al*., 1981; Manasherob *et al.,* 2003). In addition, the recommended application rate of *Bti* formulations such as VectoBac® to mosquito breeding sites is usually every 2 weeks, depending on the quality of the water body and larval density (Anon., 2004). Studies have reported that application of VectoBac® to sites where mosquitoes breed continuously would require frequent (almost weekly) application of VectoBac® (Karch *et al*., 2001; Fillinger *et al*., 2003; Anderson *et al*., 2011). Since *Bti*

formulations should be applied every 2 weeks to achieve control (Anon., 2004), *Bti* was added every alternate week in our SAM studies. Thus, *Bti* was added a total of five times during the course of the study, to ensure the presence of *Bti* in the microcosms for at least 5 weeks, in order to evaluate if any non-target organisms or other variables would be effected by *Bti*. PCC 7120#11 was only added once to the microcosms, as previous studies performed by Manasherob *et al*. (2003) reported that in outdoor experiments PCC 7120#11 toxicity persisted for 21 days. Since *Bti* was expected to persist for only a week after application, the *Bti* microcosm experiments were performed for approximately 8 weeks to account for the addition of *Bti* every alternate week, whereas the PCC 7120#11 microcosm study was performed for 5 weeks (3 weeks based on the persistence reported by Manasherob *et al*., 2003 plus an extra 2 weeks to detect any non-target effects that may occur). Although the *Bti* microcosm experiments were performed for 8 weeks, *Bti* was only present in the microcosms for a total of 5 weeks. Since the PCC 7120#11 microcosm experiments were performed for 5 weeks, the effects of PCC 7120#11 and *Bti* on the all the variables were evaluated for a similar time period (a total of 5 weeks).

One of the advantages of using the SAM protocol is that the microcosms are standardized and can be reproduced at any time (Taub, 1989; Taub, 1997). Although the control microcosms were set up at different times, the *Bti* and PCC 7120#11 control microcosms in Chapter 4 and Chapter 5, respectively, performed similarly with all the variables evaluated having very similar profiles. Thus, confirming the reproducibility of the SAM protocol.

Chapter 4 showed that the larvicidal activity of *Bti* decreased rapidly from 87% to 15% mortality within a week, which was expected when considering previous reports (Mulligan *et al.*, 1980; Davidson *et al*., 1981). Subsequent additions of the same concentration (3.5×10^4 spores/ml) of *Bti* resulted in lower larval mortality (65%), with no mortality recorded a week later (Chapter

4). The decrease in larvicidal mortality was probably due to a combination of factors including ingestion of the *Bti* particles (spores and crystals) by other invertebrates, the presence of other food sources for the mosquito larvae, and degradation of the *Bti* crystals (Blaustein & Margalit, 1991; Manasherob *et al*., 2003; Marten, 2007). The high mortality of approximately 87% after the first treatment, suggests that initially, other food sources may not have been readily available for the *An. arabiensis* larvae. As the microcosm developed, the presence of alternative food sources for the *An. arabiensis* larvae could possibly limit the amount of *Bti* ingested, thereby reducing larval mortality.

Bti was shown to be non-toxic to the non-target invertebrates evaluated in this study (Chapter 4) and did not significantly affect the other variables, such as net photosynthesis or inorganic nutrients, in the microcosms. The general trends for all the variables evaluated were similar between the control and *Bti* microcosms. The main effect of treatment was not statistically significant for any of the variables evaluated, however the interaction between treatment and time was shown to be significant only on day 56 in the *D. pulex* populations (Chapter 4). This difference cannot be attributed to the presence of *Bti* because if *Bti* had a significant effect on *Daphnia* sp. populations, the effect would have occurred much earlier as has been shown in other studies where the treatment effect occurred within days of application (Taub *et al*., 1988; Taub *et al*., 1991; Meador *et al*., 1993), rather than only at the end of a 56 day period.

Similar to what was observed in the *Bti* microcosms, the larvicidal activity of PCC 7120#11 against *An. arabiensis* larvae was shown to decrease considerably within a week from 77% to approximately 18% (Chapter 5). The reduced larvicidal mortality observed in this study could have been caused by several factors including a decrease in the concentration of PCC 7120#11, either from reduced replication and/or cell death. Although previous studies have shown that PCC 7120#11 is capable of multiplying in mosquito breeding

sites (Boussiba *et al*., 2000; Manasherob *et al*., 2002; Manasherob *et al*., 2003), certain factors in the microcosm could have limited the replication of PCC 7120#11. Under the current microcosm conditions, the PCC 7120#11 cell concentrations initially decreased before remaining at constant levels (Chapter 5), suggesting that the reduced larvicidal activity observed could be a result of a lower concentration of the PCC 7120#11 cells. The invertebrates evaluated in the PCC 7120#11 microcosms are unlikely to compete with the mosquito larvae for a filamentous cyanobacterium, especially if smaller, unicellular algae, like *C. vulgaris*, *S. capricornutum*, and *S. obliquus*, are present (Thompson *et al*., 1982). This suggests that competition for the PCC 7120#11 cells from the invertebrates evaluated in PCC 7120#11 microcosms was probably not a major factor for decreasing larval mortality. However, a likely factor for decreasing larval mortality is the ingestion of other food sources, such as unicellular algae, bacteria, and protozoans, by the *An. arabiensis* larvae. This could limit or reduce the intake of PCC 7120#11, thereby decreasing larvicidal activity.

PCC 7120#11 did not have a significant effect on the net photosynthesis, the ammonia and nitrite concentrations, or the *Coleps* sp. populations. However, evaluation of the interaction between treatment and time showed that PCC 7120#11 had a temporary significant effect on the invertebrate populations (*D. pulex*, *C. africanus,* and *Lepadella* sp.). The significant differences in the *C. africanus* and *Lepadella* sp. populations observed towards the end of the experiment (days 32 and 35; day 32, respectively) is not necessarily a direct effect of PCC 7120#11 as differences due to toxicity, are expected to occur within days of addition of treatment (Taub *et al*., 1988; Taub *et al*., 1991; Meador *et al*., 1993). Although the interaction between treatment and time on *D. pulex* populations was significant on days 18 and 25, by day 32 the *D. pulex* populations in the PCC 7120#11 microcosms were statistically identical to the populations in the control microcosms. The addition of a high concentration of PCC 7120#11 cells on day 7 however, did significantly

increase the concentration of phosphate and nitrate in the microcosms post treatment, but only for a period of not more than 4 days. The temporary increase in phosphate and nitrate concentrations did not result in an imbalance in the microcosms, as there was no significant increase in the total photosynthetic microorganism (PMO) concentration. PMOs utilize phosphates and nitrates for growth (Sterner & Hessen, 1994), thus an increase in the phosphate and nitrate concentration should result in an increase in PMOs. Furthermore, the phosphate and nitrate concentrations returned to statistically similar concentrations as those observed in the control microcosms before the end of the experiment. In addition, the presence of a high concentration of PCC 7120#11 cells in the microcosms did not result in any significant differences in the rate of inorganic nutrient depletion between the control and PCC 7120#11 microcosms (Chapter 5).

Importantly, by the end of the experiment there were no significant differences between the control and PCC 7120#11 microcosms for any of the variables that had exhibited temporary significant differences earlier in the experiment. This is important as it shows that although the PCC 7120#11 cell concentrations remained low in the microcosms for several weeks, PCC 7120#11 did not have any long-term effects on the evaluated variables and is thus unlikely to have any long-term effects on non-target organisms in the field. Chapter 5 showed that PCC 7120#11 can be considered to be an environmentally safe agent.

PCC 7120#11 could play an important role in integrated vector control programs, however before PCC 7120#11 can be utilized in any control programs or produced for commercial purposes, the large-scale production of PCC 7120#11 would have to be evaluated. Thus, the last aim of this thesis (Chapter 6) was to determine if PCC 7120#11 could be produced on a large-scale, without losing its larvicidal activity. Two major systems have been used for the large-scale cultivation of cyanobacteria; the open cultivation system

and the closed cultivation system (Posten, 2009). This study evaluated the use of closed cultivation systems, in particular indoor, closed systems, as they have several advantages over open systems including better control of the important factors (such as temperature, illuminance, and carbon dioxide), reduced contamination risks, and reproducible cultivation conditions (Borowitzka, 1997; Pulz, 2001). Several researchers use single factor optimization whereby other factors involved are maintained at a constant level, with successive changes in variables (Elibol, 2004). Single factor optimizations are tedious and are often unreliable especially as the interaction between different factors is often not evaluated (Abdel-Fattah *et al*., 2005; Bas & Boyaci, 2007; Li *et al*., 2007).

Response surface methodology (RSM) has several advantages over single factor optimizations as it is a cost-effective and time-saving technique that allows for the examination of many variables (Jacob-Lopes *et al*., 2008). Thus, Chapter 6 used RSM to design an experiment and model that would aid in evaluating the relationships between the growth and the independent variables, with the purpose of optimizing growth (Myers & Montgomery, 2002; Bas & Boyaci, 2007). RSM proved to be a very good approach in determining the complex interactions of the three key growth factors that are necessary for the optimal growth of PCC 7120#11.

The conditions that lead to the optimal production of PCC 7120#11 in the flat-plate, inclined photobioreactor were determined to be an illuminance of 5368 lux, with an air flow rate of 1.01 vvm, and carbon dioxide input level of 3.74% (v/v). The optimal VOR of 0.48 g/l/d obtained in Chapter 6 was within the range obtained in other studies that utilized photobioreactors to culture cyanobacteria (Tredici & Zittelli, 1998; Yoon *et al*., 2008). This study demonstrated that there was a complex interaction between the evaluated factors of growth. Chapter 6 showed that the percentage of carbon dioxide introduced into the photobioreactor is important in obtaining the optimal

volumetric output rate (VOR), however it cannot be considered in isolation, as both air flow and illuminance are also important in obtaining optimal VOR. The interaction of carbon dioxide and air flow and the interaction of air flow and illuminance were shown to play significant roles in the optimal growth of PCC 7120#11. These interactions may have gone undetected if single factor optimizations were performed and thus factor levels resulting in optimal growth would not have been identified. No studies, to our knowledge, have evaluated the effects of the interaction and/or combination of all three of these key factors of growth. Chapter 6 was a very important study as it allowed for the optimal selection of factors required for the optimal growth of PCC 7120#11. Furthermore, the different combinations of the key growth factors did not significantly affect the larvicidal activity of PCC 7120#11.

Large-scale production of PCC 7120#11 is crucial for any field application or vector control program. With a working volume of 3.7 litres (l), the flat-plate photobioreactor has the capacity to produce more than 4×10^{11} PCC 7120#11 cells per run (in less than a week). If one had to apply PCC 7120#11, at a concentration that is expected to cause 90% mortality of the target mosquito larvae, to a 100 l water body, one run in the photobioreactor would produce sufficient PCC 7120#11 cells. Thus, at the optimized growth conditions of PCC 7120#11 as described above, it would possible to produce amounts of PCC 7120#11 that could significantly contribute to a vector control program. In addition, significant scale-up of the flat-plate photobioreactor is possible, whereby several flat-plate bioreactors can be linked in sequence (Hu *et al*., 1996), resulting in large quantities of PCC 7120#11.

The main objective of this thesis was to evaluate PCC 7120#11 as a potential biopesticide for control of malaria vectors in southern Africa. PCC 7120#11 exhibited larvicidal activity against the majority of the anopheline vectors evaluated. The results of the PCC 7120#11 SAM study were similar to that of the *Bti* SAM study in that there were no long-term effects on non-target

invertebrates or on the chemical and physical variables evaluated. This suggests that PCC 7120#11 is an environmentally friendly agent with the potential to be an effective control agent. Although PCC 7120#11 was expected to persist longer than *Bti* in the microcosms, it appears that conditions in the microcosms may have been unfavourable for PCC 7120#11, as previous studies reported that PCC 7120#11 was larvicidal for several weeks (Manasherob *et al*., 2003). Although typical mosquito breeding sites are clear, low, turbidity water bodies (Xu *et al*., 2000), PCC 7120#11 was tested in a standardized microcosm with high abundance of PMOs and invertebrates to comprehensively evaluate the possible non-target effects of PCC 7120#11 against a range of species. However, a different persistence and larvicidal profile may be observed if PCC 7120#11 is applied to water bodies with lower concentrations of PMOs and invertebrates.

Although PCC 7120#11 was not highly larvicidal against *An. funestus*, PCC 7120#11 could be applied to areas or regions where *An. funestus* is not the predominant vector. Alternatively, PCC 7120#11 could be applied as a larvicidal agent as part of integrated vector control programs that target adult *An. funestus* mosquitoes and *An. arabiensis* larvae. In such programs, the adult *An. funestus* mosquitoes would be targeted by using indoor residual spraying or treated bed nets, as *An. funestus* mosquitoes exhibit endophilic behaviour (Coetzee & Fontenille, 2004; Okoye *et al*., 2008). The *An. arabiensis* mosquitoes exhibit variable behaviour being both endophilic and exophilic (White, 1974; Gillies & Coetzee, 1987), rendering control programs that target adults not as efficient as they could be. One efficient way of controlling *An. arabiensis* is by targeting the larval stages and the advantage of PCC 7120#11 is that it is highly larvicidal against *An. arabiensis* larvae. PCC 7120#11 could be applied to water bodies near human dwellings without causing any detrimental effects to the environment. Thus, PCC 7120#11 appears to have potential as a larvicidal agent.

7.2 Future research

This study has important implications for integrated vector control programs; however, there are several aspects that may be evaluated further to improve the assessment of the commercialization potential of PCC 7120#11. PCC 7120 #11 can be evaluated more comprehensively in different water bodies and under different conditions (such as evaluating the effect of UV) to determine its persistence. Current *Bti* formulations stipulate on the packaging that the formulations should not be applied to polluted waters such as sewage lagoons or waters containing high concentrations of algae or other particulates (Mittal, 2003; Anon., 2004). Thus, future studies may focus on determining the efficacy of PCC 7120#11 under a range of turbidites in order to establish its larvicidal activity under a range of different conditions.

Delécluse *et al.* (1993) showed that Cry4Aa was toxic to fourth instar *A. aegypti*, *C. pipiens* and third instar *An. stephensi* larvae, whereas Cry4Ba was only toxic towards *A. aegypti* and *An. stephensi* larvae. Furthermore, the larvicidal activity of a combination of both Cry4Aa and Cry4Ba was higher than that of the individual toxins, but not as high as the *Bti* crystal (Delécluse *et al.*, 1993). In addition, several studies have shown that the Cyt protein (Cyt1Aa in particular) plays an important role in larvicidal activity (Wu *et al*., 1994; Crickmore *et al*., 1995; Poncet *et al*., 1995; Pérez *et al*., 2005; Gómez *et al*., 2007; Otieno-Ayayo *et al*., 2008). Thus, further research may determine which combinations of the Cry and Cyt proteins would result in high larvicidal activity against *An. funestus* larvae.

Future studies could evaluate production of PCC 7120#11 in a flat-plate, inclined photobioreactor on an even larger scale, whereby several flat-plate photobioreactors (each with a working volume of 3.7 l) are linked in series. Such a linked system could be evaluated outdoors in an attempt to address the issue of space and reduce the cost of illuminance.

7.3 Conclusion

This thesis has contributed to the field of malaria vector control by evaluating an alternative, environmentally safe, larvicidal vector control agent and confirming the potential of *Bti* as a control agent for important anopheline vectors in southern Africa. To our knowledge, no studies have evaluated comprehensively, under the same experimental conditions, the larvicidal activity of *Bti* against the most important African anopheline species. The determination of the larvicidal activity of *Bti* is important as the larvicidal activity of *Bti* can be considered a reference point to compare the larvicidal activity of PCC 7120#11 against important malaria-carrying vectors. The confirmation, in this study, that *Bti* does not have an effect on non-target organisms further validates its use in current control programs. The SAM experiment was crucial in establishing the potential environmental risks of PCC 7120#11 on non-target organisms and other variables evaluated. The study suggests that PCC 7210#11 will not have any adverse effects on the receiving environment. The use of RSM enabled evaluation of the interaction of key growth factors and on PCC 7120#11 production (VOR) and identification of factor levels that resulted in optimal VOR.

Taking into consideration, the good larvicidal activity of PCC 7120#11 against most of the important African malaria vectors, the safety of PCC 7120#11 to non-target organisms in the SAM experiment, and the ability to mass produce PCC 7120#11, this study shows that in the long-term PCC 7120#11 has the potential to be used as a control agent as part of an integrated vector control program.

Chapter 8

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