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Toxicity and Mode of Action of the Pharmaceutical Fungicides Fluconazole and Terbinafine to Freshwater Algae

Master of Science Thesis

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Thesis work was performed at Gothenburg University in the department of Plant and Environmental Sciences

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ABSTRACT

Several antimycotics (pharmaceutical fungicides) are currently registered for use in Sweden, and are used to treat human and veterinary fungal infections perorally or topically. Fluconazole, which is an antimycotic from the imidazole group, and terbinafine, which is an allylamine antimycotic, are among the compounds currently registered. The toxicity of both terbinafine and fluconazole were studied first by conducting a single-species algal assay with the green algae, *Pseudokirchneriella subcapitata*. Terbinafine was highly toxic to the algae, with an EC_{50} (concentration required to lower growth rate by 50%) of 90 nM. The highest concentration at which there was no statistical difference from the controls (NOEC) was 10 nM, and the lowest concentration at which a statistically significant difference was observed (LOEC) was 20 nM. Fluconazole was much less toxic to the algae compared to terbinafine. The LOEC for fluconazole was 64 µM, and even at such a high concentration, the growth rate was inhibited by less than 10%. The NOEC for fluconazole was 50 µM. At the highest concentration tested, 100 µM, the percent inhibition of growth rate was approximately 12%. Both terbinafine and fluconazole inhibit fungal growth by blocking enzymes necessary for sterol biosynthesis. Terbinafine inhibits squalene epoxidase, and fluconazole inhibits cytochrome P-450-dependent 14α demethylase ($P\text{-}450_{DM}$). To determine whether the growth inhibition observed in the assay was a result of inhibition of sterol biosynthesis, sterols were extracted and analyzed using GC-MS. For terbinafine, an accumulation of squalene was observed, starting at approximately 50 nM. For fluconazole, an accumulation of 14α-sterols was observed, starting at approximately 10 µM, indicating that these drugs also influence sterol biosynthesis in algae. The toxicity of the two pharmaceuticals to natural algal communities was also assessed. Terbinafine was highly toxic to the green algae in the communities, with an EC_{50} of 20 nM. The diatoms were less sensitive to terbinafine and no effects on this algal class could be noted, even in the highest treatment, of 100 nM. Due to its toxicity to both *Pseudokirchneriella subcapitata* and green algal communities, terbinafine should be labeled as 'very toxic to aquatic life.' Both the green algae and the diatoms did not appear to be sensitive to the fluconazole treatments, even at the highest tested concentration, 100 µM. However, due to the lack of replicates associated with this data, little can be said concerning whether any trends were observed.

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LIST OF ABBREVIATIONS

Δ^7-chondrillastenol	24-ethyl-5α-cholest-7-en-3β-ol
Chondrillasterol	24-ethyl-5α-cholest,22-dien-3β-ol
DD/(DD+DT)	Measure of response of diatom light regulation pigments. Diadanoxanthin content, divided by the sum of diadanoxanthin and diatoxanthin
EC_{50}	The concentration at which 50% inhibition occurs
EC_{95}	The concentration at which 95% inhibition occurs
Ergostenol	24-methyl-5α-cholest-7-en-3β-ol
EMA	European Medicine's Agency
FSC	Forward scatter
GC/MS	Gas Chromatography Mass Spectroscopy
HPLC	High performance liquid chromatography
LC-MS-MS	Liquid chromatography coupled with tandem mass spectrometry
LOEC	Lowest observed effect concentration
LOQ	Limit of Quantification
MIC	Minimum inhibitory concentration
NOEC	No observed effect concentration
OECD	Organization for Economic Cooperation and Development
PNEC	Predicted no-effect concentration
P. Subcapitata	*Pseudokirchneriella subcapitata*
SPE	Solid-phase extraction
SSC	Side scatter
V/VAZ	Measure of response in green algal light regulation pigments. Violaxanthin content divided by the sum of violaxanthin, antheraxanthin, and zeaxanthin
WWTP	Wastewater Treatment Plant

1.0 INTRODUCTION

Several antimycotics (pharmaceutical fungicides) are currently registered for human and veterinary use in Sweden, and are used to treat fungal infections orally or topically. Fluconazole, which is an antimycotic from the imidazole group, and terbinafine, which is an allylamine antimycotic, are among the compounds currently registered. Via multiple routes, including wastewater treatment plants and septic tanks, many pharmaceuticals eventually make their way into the environment.[1]

Data on the occurrence of antimycotics in the environment is limited. Surveys of wastewater treatment plants (WWTP) for these compounds have been conducted in Sweden and in Switzerland. In Sweden, five WWTPs were surveyed. Fluconazole was detected at similar levels in the raw influent and in the treated effluent, at concentrations ranging between <5 and 140 ng/L, indicating that sewage treatment does not remove this compound. Terbinafine, was found to be effectively removed during treatment, and was detected in sewage sludge.[2] In Switzerland, ten WWTPs were surveyed in 2007 for azole fungicides. Fluconazole was detected in all of the WWTP influent streams studied, at concentrations ranging between 10 and 110 ng/L. Similarly to the study conducted in Sweden, fluconazole was detected at similar concentrations in the effluent.[3]

Freshwater environmental surveys for these compounds have only been conducted in Switzerland. In 2007, the presence of fluconazole was measured in nine lakes in Switzerland. Fluconazole was detected in the majority of the lakes sampled, at concentrations below 10 ng/L.[3] Terbinafine was not included in the study.

The effects that low levels of pharmaceuticals might cause in freshwater ecosystems are not well understood. In algae, the biosynthesis of sterols, which are lipids that help to form the cell membrane, is an essential metabolic process. Azole fungicides block sterol synthesis by inhibiting cytochrome P450-dependent 14α-demethylation.[4] Depletion of end-product sterols and accumulation of sterol intermediates, such as 14-methylated sterols, is believed to result in ceased growth and at higher concentrations cell death, primarily because of comprised cell wall integrity and function.[5,6] Terbinafine acts by inhibiting the early steps of sterol biosynthesis. Allylamines inhibit squalene epoxidase, which catalyzes squalene epoxidation. In the presence of terbinafine, squalene is accumulated and other sterol intermediates are absent.[7] In addition to a compromised cell wall and function from a depletion of end-product sterols, fungal cell death is believed to be due to the toxic effect of squalene.[8] Simply based on their mode of action, it can be hypothesized that these pharmaceutical fungicides may be toxic to freshwater micro-algal communities.

The aim of this master's thesis was to determine whether one antimycotic from the imidazole group, fluconazole, and one allylamine antimycotic, terbinafine, are toxic to freshwater algae, to compare their toxicity and to evaluate their mode of action in algae. The toxicity was first measured to one species of green algae, *Pseudokirchneriella subcapitata* (*P. subcapitata*), in the laboratory. A 72-hour assay was conducted for each compound and toxicity to both the growth rate and the total biomass of the green algae was determined by measuring the algal cell number daily using a flow cytometer. In addition, pigment samples were taken at the end of the experiment. Sterol samples were also taken at the end of each experiment, to assess changes occurring in the sterol composition. The goal was to determine whether the mode of inhibition for fluconazole and terbinafine, which had previously only been described in fungi, also exists in algae. The results from the single species algal assays were used to plan a toxicity test with natural algal communities. The natural community was sampled from the stream Mölndalsån near the town of Landvetter. Changes in the community structure were analyzed by sampling for pigments at the end of the test. Sterol samples were also taken so that the mode of action on a community level could be analyzed.

2.0 THEORY

2.1 PHARMACEUTICALS IN THE ENVIRONMENT

Pharmaceuticals enter the environment via multiple routes, as shown in Figure 1. Pharmaceuticals used to treat humans are applied topically or ingested, absorbed, metabolized, and then excreted, where they enter the sewage stream and into wastewater treatment plants (WWTP). The physical chemical properties of the compound largely determine its fate in the WWTP. Hydrophilic compounds are more likely to pass through the WWTP, while hydrophobic compounds are more likely to partition to the sewage sludge. If the sewage sludge is land-applied, these pharmaceuticals compounds may contaminate soil, and water bodies if there is runoff.[1] Pharmaceuticals can also enter wastewater treatment from the manufacturing process.

In addition, there are more direct routes of entry for pharmaceuticals in the environment. A fraction of the pharmaceuticals used to treat humans may contaminate soil and ground water if septic systems are used. If pharmaceuticals, such as antibiotics, are used to prevent illness and disease to fish in aquaculture, a portion of those compounds will be directly discharged to water bodies. In the USA, between approximately 92,500 and 196,400 kg of antibacterials per year are used in aquaculture.[1] Pharmaceuticals are used for similar purposes with livestock. Livestock waste, which contains low-levels of pharmaceuticals or their degradation products, is generally land-applied, and can contaminate both soil and fresh-water bodies. In the USA, between approximately 8.5 and 11.2 million kg of antibacterials are used to treat livestock annually.[1] Lastly, pharmaceuticals used to treat family pets, can contaminate the environment if pet waste is discharged to soil.

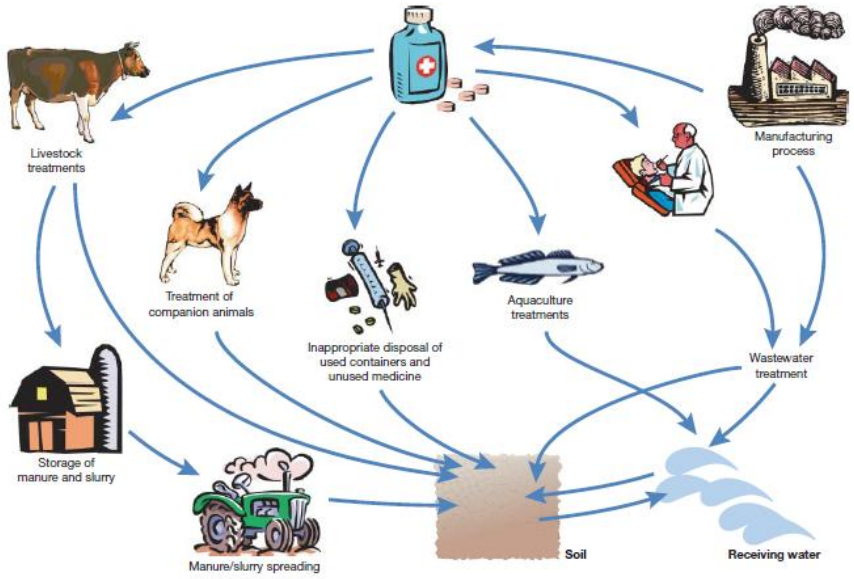

Figure 1. Illustration of different pathways, by which pharmaceuticals contaminate soil and water.[1]

The concentrations of pharmaceuticals in freshwater and saltwater bodies are not well quantified, due to an insufficient number of surveys, and historically, due to poor analytical techniques. However, due to the development of analytics such as liquid chromatography coupled with tandem mass spectrometry (LC-MS-MS), detection limits are lower and a growing number of studies quantify concentrations of chemicals in wastewater treatment plants, soils, surface waters, and ground water.[1]

2.2 TERBINAFINE AND FLUCONAZOLE IN THE ENVIRONMENT

The presence of terbinafine and fluconazole in both WWTP influent, effluent, and sewage sludge was investigated at five Swedish WWTPs in 2007.[2] The WWTPs sampled were Stockholm (Henriksdal), Gothenburg (Ryaverket), Umeå, Alingsås (Nolhaga), and Bollebygd. These WWTPs differ in both size and catchment area. [2]

Terbinafine was detected below the limit of quantification (LOQ) in both the raw sewage water and the final effluent at all five WWTPs. In the digested, dewatered sludge, terbinafine was detected at concentrations ranging between 4 and 30 µg/kg at three of the WWTPs. At the other two WWTPs, terbinafine was detected below the LOQ.[2]

Fluconazole was detected above the LOQ in the raw sewage water at one of the WWTPs tested, at a concentration of 90 ng/L. Fluconazole was detected above the LOQ in the final effluent at two of the WWTPs tested, at concentrations of 140 and 100 ng/L. Fluconazole was not detected above the LOQ in the digested sewage sludge at any of the WWTPs tested. Sewage water was also collected from the Umeå County Hospital during this time-period, and fluconazole was detected, at a concentration of 570 ng/L.[2]

In addition, raw sewage water, raw sewage water particles, raw sludge, and digested dewatered sludge was collected from the Umeå WWTP in April 2008 to assess the fate of the chemicals during the treatment process. Terbinafine was not detected in the raw sewage water or the final effluent; however, it was detected in the raw sludge, at a concentration of 40 µg/kg. Terbinafine appears to be effectively removed from the water during treatment. However, as discussed in Section 2.1, if the sewage sludge is land applied, terbinafine may still enter the environment. Fluconazole was detected at similar levels in the influent and effluent, at concentrations of 120 and 100 ng/L, respectively. Fluconazole was not detected above the LOQ in the raw sewage particles, raw sludge, or the digested dewatered sludge. It was the only compound investigated that passes directly through the WWTP. [2]

In Sweden, approximately 43 kg of terbinafine, and 121 kg of fluconazole is used per year. These figures are based on Apoteket AB's sales during 2007.[2] Based on the Swedish sales, in combination with the daily defined dose, the predicted environmental concentration in surface water was calculated to be 6 ng/L for terbinafine according to the European Medicine's Agency (EMA) guidelines Phase I.[36] However, the EMA guidelines assume that retention in sewage sludge does not occur, so based on the previously discussed findings, this estimate is likely too high. If the mean mass flow values of terbinafine through the WWTPs are normalized to the number of people connected to the WWTP, 1% of the terbinafine used appears in the digested dewatered sewage sludge. This may be due to the fact that terbinafine is generally administered topically and so only a small percentage of the drug is actually discharged to the WWTP.[2]

Based on the Swedish sales value for fluconazole, in combination with the daily defined dose, the predicted environmental concentration in surface water was calculated to be 20 ng/L (0.065 nM), according to the EMA. Fluconazole is believed to be persistent in the environment.[9] Approximately 80% of the dose taken perorally is excreted in the urine. If the mean mass flow values of fluconazole through the WWTPs are normalized to the number of people connected to the WWTP, 53% of the purchased fluconazole appears in the final effluent.[2]

In Switzerland, wastewater samples were sampled for azole fungicides from ten WWTPs located in the Canton of Zürich.[3] These WWTPs serve between 10,000 and 370,000 people. Fluconazole was detected in all of the WWTP influents, at concentrations ranging from 32 to 109 ng/L. Fluconazole was detected at

similar levels in all of the WWTP effluents, at concentrations ranging from 28 to 83 ng/L. For a one week period, influent and effluent concentrations were monitored at the WWTP in Zürich. Fluconazole concentrations were consistent with those measured previously in both the influent and the effluent, providing further evidence that fluconazole is not removed during wastewater treatment.[3]

In addition, surface water from nine Swiss Midland lakes (Walensee, Vierwaldstättersee, Zürichsee, Sempachersee, Hallwilersee, Zugersee, Baldeggersee, Pfäffikersee, and Greifensee) was sampled, at the outflow of each lake. Fluconazole was detected in six of the lakes, in the low ng/L range (<10 ng/L).[3]

A summary of the environmental data described in this section for fluconazole and terbinafine, converted to nM concentrations (and nM/kg, where appropriate), is included in Table One.

Table 1
Summary of Data on the Occurrence of Fluconazole and Terbinafine in the Environment

Location	*Concentration*
Terbinafine	
Sewage Sludge	31 nM/kg[2]
Fluconazole	
Wastewater Treatment Plant Influent	0.1 - 0.39 nM[2,3]
Wastewater Treatment Plant Effluent	0.09 - 0.46 nM[2,3]
Hospital Sewage Water	1.9 nM[2,3]
Lake Surface Water	<0.03 nM[3]

2.3 Single-Species Algal Assays

Algae play an important role in primary production and energy-cycling worldwide. Algae grow in the phototrophic zone, i.e. where the light is able to penetrate, in all of the world's oceans and seas, which cover approximately two-thirds of the planet. They are also present on shores and coasts, and in freshwater bodies. Algae account for approximately fifty percent of the primary production in the world. Especially aquatic organisms depend upon the solar energy captured by algae.[9] Thus the functions of algal communities is critical to the health of ecosystems.

When pollutants are introduced into an ecosystem the response by the organisms living within the ecosystem is species-specific. There is no single species that can be considered representative of how all the organisms in the ecosystem will respond. Toxicity results for invertebrates and fish were historically considered to be representative for algae, but this is no longer considered valid. Algae have been found to be more sensitive than invertebrates and fish to several heavy metals, pulp mill and industrial effluents, pesticides, and other chemicals.[10] Thus, algal toxicity tests are necessary for the hazard and risk assessment of chemicals.

Single-species algal assays are relatively easy to perform in the laboratory and are inexpensive. Static tests are conducted over a time-period ranging from 72 to 96 hours with an exponentially growing algal population. Although these tests are considered acute (short-term) from a regulatory perspective[11], it can be argued that they are chronic (long-term) because the effects are assessed over several generations. The tests are conducted under conditions of controlled temperature, light and initial pH. Filtered oxygen and carbon dioxide are bubbled through the test-medium. Species for testing are selected primarily on availability and ease of culturing.

Pseudokirchneriella subcapitata (*P. subcapitata*), a green algae, is one of the most frequently used species. *P. subcapitata* belongs to the algal class, Chlorophyceae. *P. subcapita* is kidney shaped, as shown in Figure 2. This organism was first isolated from the river Nitelva in Norway in 1959.[12] The organism was originally mischaracterized as a *Selenastrum*, and was named *Selenastrum capricornutum.* However, as the organism is not actually a true *Selenastrum*, but is more closely related to *Kirchneriella*, it was renamed as *Pseudokirchneriella subcapitata*.[12]

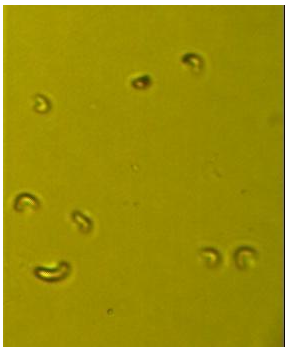

Figure 2. Picture of *Pseudokirchneriella subcapitata*, taken with a microscope.

Freshwater blue-green algae and diatoms are used less frequently than *P. subcapitata* and other species of green algae because they are more difficult to culture. However, the difference in toxicity between these species can vary by several orders of magnitude so the toxicity of compounds to blue-green algae and diatoms should be measured as well.[10]

The sterol composition of *P. subcapitata* is reported to consist predominately of three sterols, all of which are phytosterols (which, as opposed to e.g. cholesterol, contains a methyl or ethyl group in the side chain): 24-methyl-5α-cholest-7-en-3β-ol (ergostenol), 24-ethyl-5α-cholest-7-en-3β-ol (Δ^7-chondrillastenol), and 24-ethyl-5α-cholest,22-dien-3β-ol (chondrillasterol). These three sterols comprise approximately 95% of the total sterol composition of *P. subcapitata*. Δ^7-chondrillastenol, has been reported to be the most dominant sterol, accounting for approximately 65% of the sterol composition; ergostenol and chondrillaterol comprise approximately 25% and 15%, respectively, of the total sterol content.[13]

Pollutants can have several effects on algae, including growth inhibition, growth stimulation, morphological and physiological changes. The effects of growth inhibition and growth stimulation can be measured by comparing either the growth rate or final biomass to that of the controls (see equations at the end of the section). The algal cell number can be determined by counting the number of cells in a defined volume, using for example a microscope, or flow-cytometer. The algal cell number and biomass can also be indirectly estimated by monitoring cellular constituents such as chlorophyll *a*; however, if the toxicant induces physiological or morphological changes such as increased cell-size, this may not be an accurate indicator of the algal cell number.[10]

There are several ways to present the results of an algal toxicity test. The parameters most frequently used are the concentration which causes 50% inhibition (EC_{50}) and the lowest concentration in which no statistically significant effect is observed (NOEC). The relevance of these parameters for predicting an environmentally safe level (a so-called PNEC, predicted no effect concentration) is currently debated.[10] Reports of significant inter- and intra-laboratory variation with reference toxicants are one of the reasons for the debate.[10,32,33] In the new guidelines (OECD 2006 and ISO 8692), the use of growth rate, rather than biomass to calculate end-

point parameters is recommended as a means to reduce this variation.[31,32] Furthermore, a PNEC has to consider the much higher biological, physical-chemical and spatiotemporal complexity of ecosystems compared to laboratory single species toxicity assays. For estimating, the quality of single species toxicity data available (number of species from different trophic levels, acute or chronic exposure, etc.), determines the size of the extrapolation. As an example from the European Technical Guidance document, if only acute toxicity data for one freshwater algal species is at hand, the observed EC_{50} is divided with a factor of 10,000 for establishing a PNEC for the marine environment. [14]

The average growth rate for a specific period during the test is calculated according to the following equation:

$$\mu_{i-j} = \frac{\ln(x_j) - \ln(x_i)}{t_j - t_i} (day^{-1})$$

Where:
μ_{i-j} is the average specific growth rate from i to j;
X_i is the biomass at time i;
X_j is the biomass at time j

The percent inhibition of growth rate is calculated from each treatment replicate according to the following equation:

$$\%I_r = \frac{\mu_c - \mu_T}{\mu_c} * 100$$

Where:
$\%I_r$ is the percent inhibition of the average specific growth rate;
μ_C is the arithmetic mean for the average specific growth rate (μ) in the control group;
μ_T is the arithmetic mean for the specific growth rate for the treatment replicate

And finally, the percent inhibition of biomass (cell number multiplied by the volume of the media) is calculated from each treatment replicate according to the following equation:

$$\%I_B = \frac{B_c - B_T}{B_c} * 100$$

Where:
$\%I_b$ is the percent inhibition of cell number;
B_C is the mean biomass value for the control group;
B_T is the mean biomass value for the treatment replicate

2.4 Periphyton Communities and SWIFT Tests

In freshwater bodies, numerous species of algae coexist together, and also with other micro-organisms, such as bacteria, fungi, protozoa, and metazoan. Microbial communities, consisting of a range of autotrophic and heterotrophic species, which attach to submerged surfaces, together with the polysaccharides excreted from the microalgae and other matter from the water column, are called periphyton.[15 and references therein] The community is dynamic, and the number of species, and their relative abundance, may change depending upon the season and other environmental factors. Periphyton communities contain many species which are not traditionally used in laboratory toxicity assays; however, these communities are of importance due to their primary production, cycling of nutrients, and removal of pollutants from the water column.[10] Hence, they

represent ecological entities of high environmental relevance for the environmental risk assessment of chemicals.

It would be incorrect to assume that all of the species, or even all of the algal species, are sensitive at the same concentration to a chemical. The testing of a chemical on a community level provides a more realistic picture of the ecological consequences. Compared to a single species assay, the inclusion of a greater number of species in the test provides a better representation of the natural distribution of species sensitivities. Furthermore, the organisms are exposed under more realistic conditions and as they have to manage additional, natural stressors such as predation and competition. This means that for example sublethal, but ecologically important, effects from toxicant exposure have an influence on the outcome. Moreover, because species are inter-dependant, the community structure will change when the most sensitive species is affected by a toxicant. Thus, community structure can be used as an ecotoxicological endpoint which is overarching, and independent of the biochemical mode of action of the toxicant. This in the sense that it responds to any toxicant-induced change that affects the ecological performance of the different species in the community.[16]

Microorganisms can be studied on a community level relatively easily, due to their short generation times and size. The succession in a periphyton community is similar to that of higher communities, with the favorable exception that the succession occurs at a faster rate. The effects of toxicants on periphyton communities have been tested in several different ways. In the field, assays have been conducted using experimental streams, floating exposure units, and enclosures. In the laboratory, assays have been conducted using laboratory microcosms, which is a small-scale ecosystem, and with a mixed flask culture, which uses multiple organisms taken from a natural source.[10] In addition, periphyton has been tested with flow-through microcosms in the laboratory. However, each of these setups are laborious, and it is difficult to feasibly test a range of concentrations and have sufficient replicates.

The SWIFT periphyton test employs biofilms, which have been grown on an artificial substratum submerged into the phototrophic zone of aquatic ecosystems. [16] The biofilms are introduced into a semi-static test regime, which has been inoculated with the toxicant. During the course of the four day test, artificial nutrients are added to ensure that the organisms have a high activity and a succession occurs. At the end of the four-day exposure, several endpoints can be measured. While microscopic cell counts are a traditional way of analyzing the change in community structure, identifying and counting species is very time-consuming and requires an experienced taxonomist. One faster alternative is to extract the pigments from the whole community and analyze them using high-performance liquid chromatography (HPLC). Chlorophyll *a* is often used as an indicator of total biomass, but as all microalgal species contain chlorophyll *a*, it cannot be used as an indicator for changes in community structure. However, the amount and ratios of 'marker pigments', such as chlorophyll b, fucoxanthin, and zeaxanthin provide a better resolution as they are limited to fewer algal classes. The changes in pigment profile are indicative of changes in the species composition, biomass and the physiological status of the community.[16] Xanthophylls are light protection pigments, the cellular concentrations of which are modified in response to the light regime, but may also respond to toxicant induced stress, particularly oxidative stress and/or if the photosynthetic apparatus is affected.[16] In green algae, the xantophyll pigments are violaxanthin, antheraxanthin, and zeaxanthin, whereas diatoms have diadinoxanthin and diatoxanthin. In addition, sterols can be extracted at the end of the test, to determine whether the mode of action that was observed on a single-species level is also observed on a community level.

2.5 Fungicide Classification and the Mode of Action of Terbinafine and Fluconazole

There are four major classes of antimycotic agents: polyene antibiotics, azole derivatives, morpholines, and allylamines. [17]

Azole antimycotics were first developed in the 1960s.[17] Azoles are classified by the number of nitrogen atoms located in the azole, nitrogen heterocyclic ring: the imidazoles have two nitrogens, and include ketoconazole, miconazole, and clotrimazole; the triazoles have three nitrogens and include fluconazole.[7] The structure of fluconazole is shown in Figure 3. Fluconazole is prescribed for certain systemic fungal infections, and is administered perorally.[18] As of 1999, fluconazole had been used to treat over 16 million patients, including more than 300,000 AIDS patients in the US alone. [7]

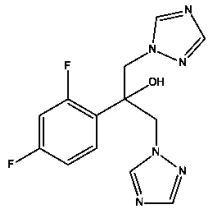

Figure 3. The molecular structure of fluconazole.

The mode of action of fluconazole is inhibition of cytochrome P-450-dependent 14α sterol demethylase (P-450_{DM}).[19] Fungi that have been exposed to fluconazole are depleted of ergosterol, and have an accumulation of 14 α-methylated sterols, such as lanosterol. The presence of lanosterol likely disrupts membrane structure and function, which results in inhibition of growth and morphogenesis.[19] One direct effect is that chitin synthase activity is adversely affected, which causes abnormal deposition of chitin in the fungal cell wall. In addition, ergosterol is believed to "spark" the cell by having a regulatory function in mitotic division. It is believed that there is an ergosterol-dependent temporal stimulation of polyphosphoinositide metabolism and phospholipid biosynthesis before yeast cells exit from phase G in the division cycle, which is disrupted in the presence of fluconazole. Fluconazole-treated *C. albicans* cells are multinucleated and abnormally large. Electron microscope studies have shown that this cell enlargement is mainly due to wall thickening, and although there are not any effects on plasma membrane structure, lipid bodies accumulate in the cytoplasm.[19]

Allylamine antimycotics, are categorized by their mode of action. The structure of terbinafine, which is an allylamine, is shown in Figure 4. Similar to azole antimycotics, allylamine antimycotics also inhibit sterol biosynthesis, but much early in the synthesis pathway, as shown in Figure 5. Terbinafine is a reversible, non-competitive inhibitor of squalene epoxidase, which together with (2,3)-oxidosqualene cyclase is responsible for the cyclization of squalene to lanosterol.[18] In the presence of terbinafine, squalene is accumulated, and sterol intermediates and end-product sterols are depleted.[7,34] Terbinafine is primarily fungicidal.[21,34] This effect is believed to result from the accumulation of toxic, intracellular concentrations of squalene.[7,18,34] The mechanism of the toxic effects of squalene are not well understood; however, it has been hypothesized that intracellular squalene may dray lipids out of the membrane, thereby influencing membrane permeability.[7,34] Terbinafine also has a fungistatic effect, which is believed to be due to the corresponding decrease in ergosterol.[34]

Terbinafine was first synthesized in the 1990s,[17,20] and is used to treat both local, and certain systemic fungal infections. [18,21]

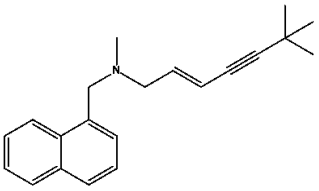

Figure 4. The molecular structure of terbinafine.

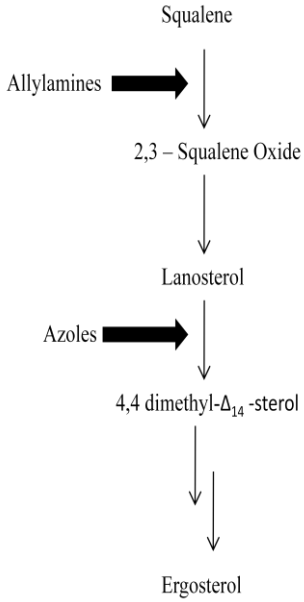

Figure 5. In fungi, ergosterol is the end-product sterol. Allylamines and azoles inhibit sterol biosynthesis at different steps in the biosynthesis chain, as shown.

2.6 Sterol Synthesis and Function

Sterols are found in all eukaryotic organisms, and are synthesized *de novo* by algae, most fungi, vertebrates, and higher plants.[22] Animals synthesize one sterol, cholesterol, while plant cells synthesize several different sterols, in genetically defined proportions. There is considerable variation in sterols between fungi, algae, higher plants, and marine organisms, and approximately 200 different sterols have been identified.[23] In addition, there are differences in the synthesis pathway between animals and plants.[24]

Sterols are primary metabolites, which are synthesized by the isoprenoid metabolism.[24] The basic sterol structure and numbering system is shown in Figure 6. All naturally occurring sterols have structural similarities; they all have between 27 and 30 carbon atoms, an oxygen atom at the C-3 position, and a side chain, which consists of at least seven carbon atoms at the C-17 position. Sterols exhibit variation in the ring structure, number of carbon atoms in the side chain, stereochemistry (particularly at the C-5 position), the number and location of double bonds, and the presence of methyl groups (generally at the C-4, C-14 or C-19 positions).[25] Both the numbering system as recommended by IUPAC-IUB in 1976 and the revision to the original numbering system in 1989, are provided in Figure 6. Many sterols have been given trivial names, which generally derive from the name of the original source. The trivial names do not provide any

information about the sterol structure; however, this naming system is more commonly used than the IUPAC-IUB numbering system.[23]

Figure 6. The basic sterol structure. The original numbering system, as recommended by IUPAC-IUB in 1976 is shown on the left. The revision to the original number system, as recommended by the IUPAC-IUB in 1989, is shown on the right.[23]

Squalene is a precursor to all sterols, and the biosynthesis of this compound is common to animals and plants. Squalene is synthesized from acetyl CoA through the universal isoprenoid route in the endoplasmic reticulum. [26] The biosynthesis pathway of squalene is shown in Figure 7.

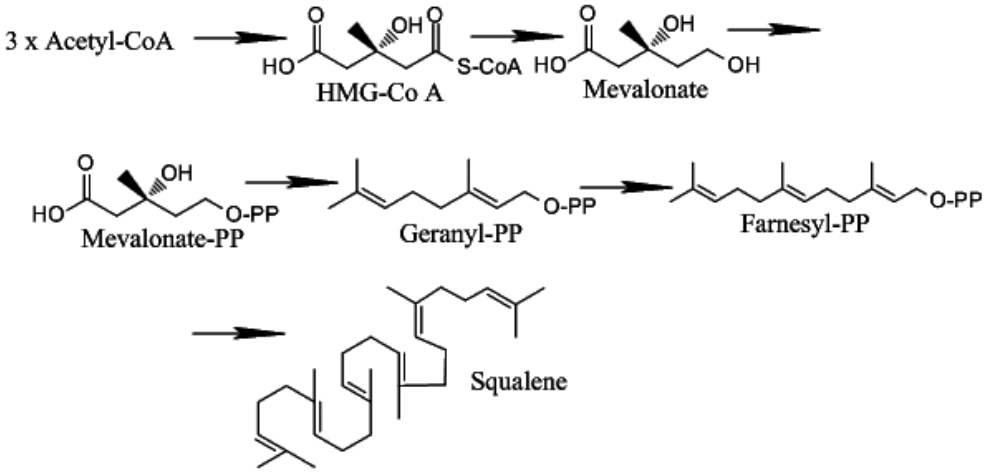

Figure 7. The biosynthetic pathway of squalene, starting from acetyl-CoA.

Squalene is then cyclized to form 3β-hydroxy-pentacyclic and tetracyclic triterpenols and sterols. With the exception of some plants, squalene must be epoxidized to yield 2,3-oxidosqualene prior to cyclization in all organisms. The first cyclised product in animals and fungi is lanosterol which is then modified to yield cholesterol in animals and ergosterol in fungi, as shown in Figure 8. In photosynthetic eukaryotes, such as algae and higher vascular plants, squalene can be cyclised into a wide variety of pentacyclic and tertracyclic triterpenols.[23] The formation of plant sterols involves over 30 enzyme-catalysed reactions.[27] Figure 9 shows the general pathway of sterol synthesis in photosynthetic eurkaryotes. As can be seen in the pathway, due to modifications of the ring system and side chain during synthesis, many sterol structures can be produced, and 1500 sterol structures are theoretically possible.[23] Plant sterols can be divided into 4-desmethyl sterols, 4α-monomethyl sterols, and 4,4-dimethyl sterols, where 4α-monomethyl sterols, and 4,4-dimethyl sterols are

sterol precursors.[27] Alkylation at the 24 position is specific to plants, and the substitution can have α or β chirality. In addition, the 3-hydroxyl group of the free sterols can be esterified by a fatty acid or a phenolic acid, yielding sterol esters.[27]

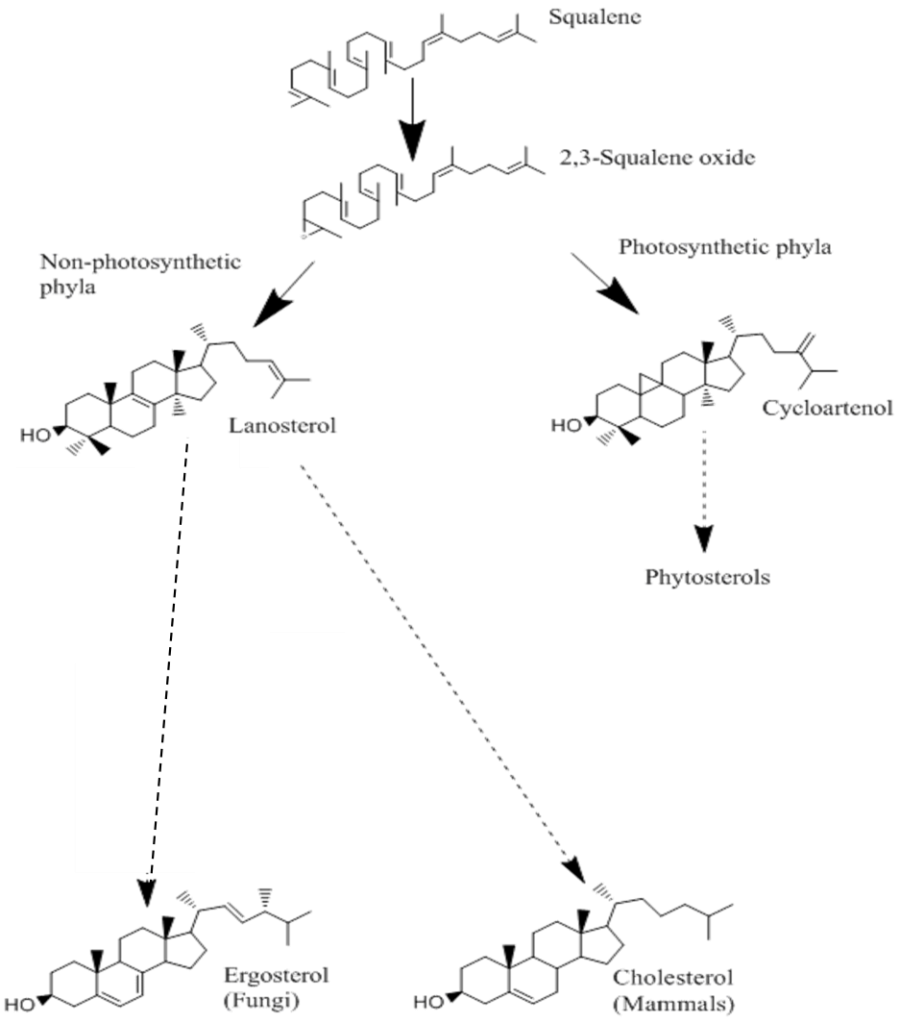

Figure 8. Simplified schematic of the synthesis pathway for sterols in mammals, fungi, and photosynthetic organisms. [23]

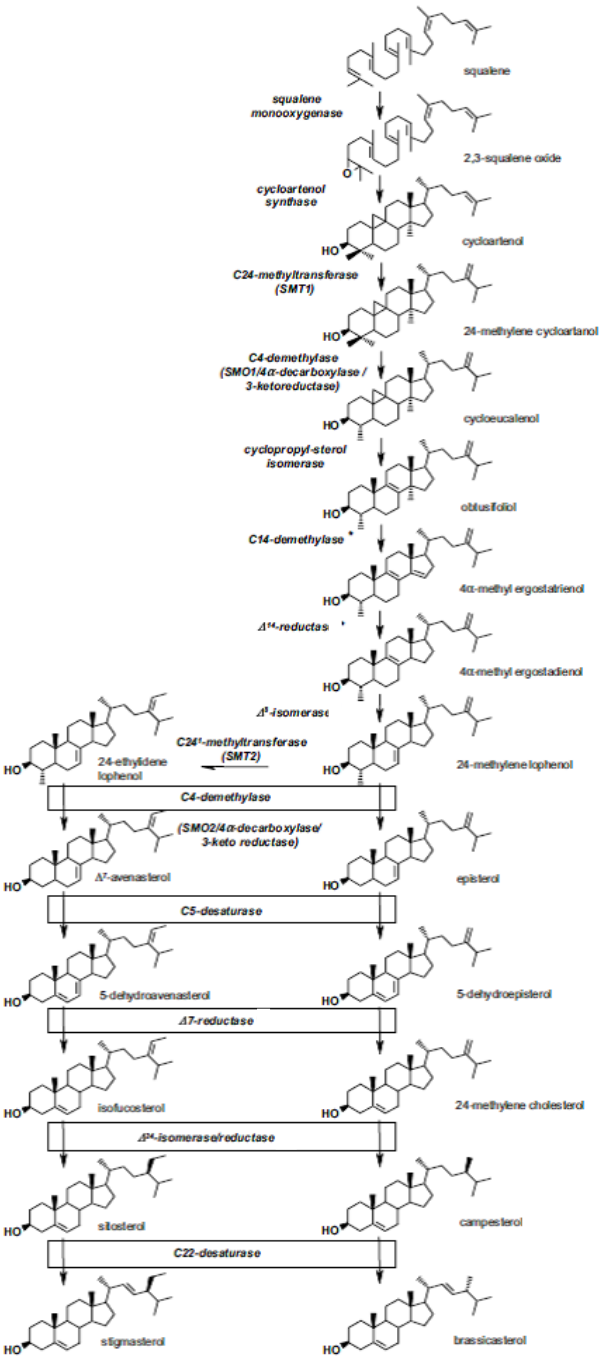

Figure 9. Depiction of the synthesis pathway of sterols in photosynthetic organisms.[24] Reactions in boxes are for both the 24-methyl(ene) and 24-ethyl(idene) segments of the pathyway.

In both plant and animal cells, sterols are an essential component of cell membranes. Sterols have approximately the same length as the phospholipid monolayer, and serve to reinforce the membrane, by restricting the motion of fatty acyl chains. Sterols are also believed to help membranes adapt to changes in

temperature, regulate the activity of membrane-associated proteins, and influence water permeability.[27] Sterols also regulate the activity of membrane-associated proteins.[24,27] In plant cells, the plasma membrane has the greatest sterol content (µg per mg protein) and the highest sterol:phospholipid ratio. Sterols are essential for plant growth and development e.g., as precursors to steroids.[27] In addition, sterols are required to form the membranes of dividing cells. Thus, if sterol production is inhibited, cell division will stop by this mechanism as well.[23] Finally, sterols have also been shown to be substrates for secondary metabolites, such as glycoalkaloids and saponins.[27]

3.0 MATERIALS AND EXPERIMENTATION/METHOD

3.1 TEST ORGANISMS AND CULTURE CONDITIONS

Liquid cultures of the unicellular green algae *Pseudokirchneriella subcapitata*, strain 61.81(Sammlung für Algenkulturen (SAG) Göttingen, Germany) were grown in Organization for Economic Cooperation and Development (OECD) medium, as outlined in the OECD Guidelines for the Testing of Chemicals 201 (2006) (OECD Guideline 201). The algae were grown in an aquarium, with water temperature maintained at 22 ± 1°C. The algae received continuous light for 16 hours and complete darkness for 8 hours. Stock culture and pre-culture were prepared as described in the OECD Guideline 201.

3.2 GROWTH INHIBITION TEST

The growth inhibition assays were prepared according to the OECD Guideline 201. Stock solutions of terbinafine and fluconazole were prepared in acetone and methanol, respectively. The volume of stock solution necessary to obtain the desired algal cell number in 65 ml of OECD media was pipetted into a 100 ml glass jar (the amount pipetted was always between 20 and 100 µl), the solvent was allowed to evaporate, and 65 ml of OECD media was subsequently added. Each concentration was prepared in triplicate and each test included three controls. The jars were shaken overnight. The OECD media, with dissolved pharmaceutical, was transferred to 90 ml test tubes and algal culture was added to obtain the desired initial cell density. Other incubation conditions were the same as was used for cultivation. The tests run in the fall 2009 were conducted over 96 hours and the initial cell density was 10,000 cells/ml. The tests run in the spring 2009 were conducted over 72 hours and the initial cell density was 20,000 cells/ml. The algal growth in each sample was determined approximately every 24 hours by measuring the cell count.

3.3 DETERMINATION OF CELL COUNT

The cell count was determined using a FACSCalibur flow cytometer (BD, Heidelberg, Germany) equipped with two lasers, an argon ion laser emitting at 488 nm, and a red diode laser emitting at 635 nm, for detection of forward-scatter (FSC), side-scatter (SSC) and three fluorescence parameters. Due to a variable flow-rate, the count measured by the flow cytometer was found to be inaccurate when compared to a manual count using a microscope (Figure 1). The count rate was therefore correlated to algal cell number by first correlating the count rate of a standardized count standard (Flow Cytometry Absolute Count Standard – Full Spectrum from Bangs Laboratories, Inc.) to a diluted sample (2,500x) of un-standardized fluorescent microspheres (Flucresbrite™ Carboxylate YG1.5 Micron Microspheres (2.5% Solids-Latex) from Polysciences, Inc.). The standardized count standard has a known concentration of 1,000,000 microspheres/mL. 100 µl of the diluted un-standardized fluorescent microspheres was subsequently added to each sample prior to analysis. The flow cytometer was found to have a low precision (measured variation as high as ~19%) at algal cell numbers exceeding 3,000,000 (Figure 10). Any samples with algal cell numbers believed/measured to be above this threshold were therefore diluted with OECD media prior to analysis. Each sample was run for 3 minutes on low flow. A computer equipped with CellQuest Pro and Worklist Manager (BD, San Jose, USA) was used for data acquisition.

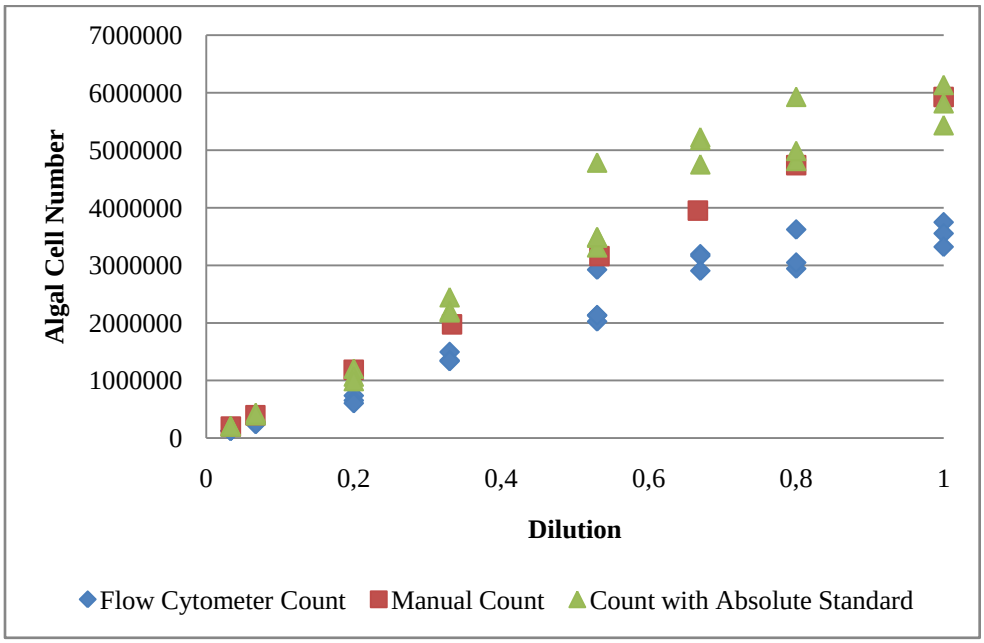

Figure 10. Determination of the accuracy of the flow cytometer. A sample of algae, which was also manually counted, was diluted seven times and the count measured on the flow cytometer. The count was also measured for each sample with an added internal standard.

3.4 Extraction and GC/MS analysis of sterols

Sterols were extracted following a method that had been developed from the chloroform-methanol protocol.[28 and 29] For the samples from the single-species assays, 24 ml of sample was centrifuged at 3300 rpm for 10 minutes, and the supernatant was discarded. For the SWIFT assays, 7 glass discs were placed in a scintillation vial, after excess water was removed with a tissue. The protocol for both sets of samples was the same. 2-isopropanol and cholestinol (for use as an internal standard) was added and the solution was boiled for five minutes. The 2-isopropanol was evaporated under nitrogen gas in a 35 to 40˚C heat-plate. 4.4 ml of chloroform:methanol:water (1:2:0.8) was added and the sample was ultrasonicated for 30 minutes, then placed in the dark at 4˚C for 30 minutes. 1.2 ml of chloroform with 0.05% BHT and 1.2 ml of 1.6 M HCl was added; the sample was vortexed, and the phases were allowed to separate. The lower chloroform phase was transferred to a new test tube (Tube 2). 2.4 ml of chloroform with 0.05% BHT was added to the original tube (Tube 1); the sample was vortexed and the phases were allowed to separate. The lower phase was again transferred to Tube 2. 2mL of methanol:HCl (1M) (1:1) was added to Tube 2. Tube 2 was vortexed and the layers were allowed to separate. The lower phase of Tube 2 was transferred to a new test tube (Tube 3). 2.4 ml of chloroform was added to Tube 2, the sample was vortexed and the layers were allowed separate. The lower phase was transferred to Tube 3. The pooled chloroform phases in Tube 3 were evaporated under nitrogen gas in a 35 to 40˚C heat-plate; 150 µl of chloroform with 0.05% BHT was added, nitrogen gas was added, the tubes were sealed, and stored at -18 °C if necessary.

A solid-phase extraction (SPE) column (Discover SPE, 100 mg DSC-Si) was pre-conditioned with 3x1ml chloroform. The sample was added to the column and the sample test tube was rinsed with 100 µL of chloroform, which was subsequently added to the column. The sample was allowed to enter the column completely. The sterols (neutral lipids) were eluted with 4x1ml of chloroform with 10% acetone. The elutant

was evaporated under nitrogen gas in a 35 to 40°C heat-plate. 75 µL of chloroform was added and the solution was transferred to an inset in a microvial. The sample was again evaporated under nitrogen gas in a 35 to 40°C heat-plate. The sample was reconstituted in 30 µL acetonitrile and 5 µL of BSTFA was added. The samples were stored at -18 C until analysis.

Samples were analyzed in full scan mode (m/e 40-560) in a Hewlett Packard 5890 series II gas chromatograph equipped with a Hewlett Packard 5970 series mass selective detector. A DB-5 capillary column (30m x 0.25mm, J&W Scientific, Inc., Folsom, USA) was used with helium as carrier gas with a constant flow of 2 ml/min. Both injector and detector were maintained at 300°C. The oven temperature was increased from 200 to 300 C at 20°C/min. Final temperature was maintained for 15 minutes. [28,29]

Sterols were tentatively identified using the NIST98 library (Scientific Instrument Services, Inc., Ringoes, USA) and/or comparisons with published data for fragmentation patterns and retention times[25,30].

3.4 Extraction and HPLC analysis of pigments

Pigments were extracted in 2 mL of a mixture of methanol:acetone:dimethylformamide:water (30:30:30:10) under -18 °C for 2 to 7 days. In addition, periphyton community samples from the SWIFT test were ultrasonicated for 30 seconds in order to facilitate pigment extraction. Prior to injection (100 µL) in the HPLC (ThermoQuest, Thermo Scientific, equipped with a diode-array detector) samples were filtered (0.45 micron) and diluted with water (400 µL of sample + 100 µL of water). Pigments were separated using a Kinetex Ultracarb 3u ODS (20) 150 x 3.2 mm column (Phenomenex). A gradient consisting of three mobile phases was used: A: methanol:0.5 M ammonium acetate (80:20), B: acetonitrile:water (90:10), and C: ethylacetate. Pigments were identified using wavelength spectra and relative retention times. Pigment peak areas in the chromatograms were determined at 436 nm, and were used for effect assessments without further transformation.

3.5 SWIFT Tests

Glass discs (1.5 cm^2) were mounted on both sides of polyethylene sampling racks holding 170 discs each. The discs were disinfected using 70% ethanol immediately before being submerged at the sampling site. The sampling racks were suspended at a depth of approximately 0.5 meters in an approximately 1 meter deep stream (Mölndalsån) in Landvetter, Sweden. After nine days, a visible biofilm had established on the discs, which were then transported to the laboratory in a container filled with river water and shielded from direct sunlight. The discs were visually analyzed and those with atypical appearance or damage were discarded.

Twelve periphyton discs were placed in 10x15x5 cm glass containers each containing 200 mL of test medium. The containers were covered with a transparent glass lid and placed on a shaker in a climate-controlled room with continuous illumination from fluorescent tubes. The temperature in the room was set to correspond to the temperature in the water at the sampling site (10 °C). The test medium was exchanged every 24-hours during the four day test. At the end of the experiment, 5 discs were sampled for pigments and 7 discs were sampled for sterols.

The test medium consisted of river water collected one day before the start of the test from a depth of approximately 0.5 m. The water was filtered (GF/F) and stored in the climate controlled room. Solutions for testing were prepared approximately 24 hours prior to each daily replacement. Macro-and micro-nutrients were added (see Appendix One). Toxicant stock solutions were prepared in methanol. 20 µL of the toxicant was pipetted into bottles, the solvent was allowed to evaporate, and the toxicant was re-dissolved in 200 mL

of the nutrient-enriched test medium. Six incubation vessels received no toxicants and were used as controls. The bottles were stored overnight, on a shaker in the climate controlled room.

4.0 RESULTS

4.1 SINGLE SPECIES ASSAYS – BIOMASS MEASUREMENTS

4.1.1 RANGE-FINDING EXPERIMENTS: FALL 2009

A range-finding experiment for terbinafine and fluconazole was conducted in October 2009. *P. subcapitata* was exposed to eight concentrations, ranging from 32 to 0.0001 µM, of each drug, separately. As the purpose of the experiment was only to estimate the toxicity range, only one replicate for each concentration was used. Three controls were also included. The algal cell number was measured each day using the flow cytometer. For this experiment, unlike as described in Section 3.0, an internal standard was not used. Instead, the mass of the sample was weighed before and after analysis, to determine the rate of the flow of the sample through the flow cytometer. The accuracy of the flow cytometer was measured at the end of the experiment by conducting a manual cell count. For terbinafine, the 1, 10 and 32 µM treatments were not counted because the algal cell number was too low. As the manual count was found to be significantly different (and not in a predictable way) from the flow cytometer count, as shown in Figures 11 and 12, an internal standard was used in all subsequent experiments.

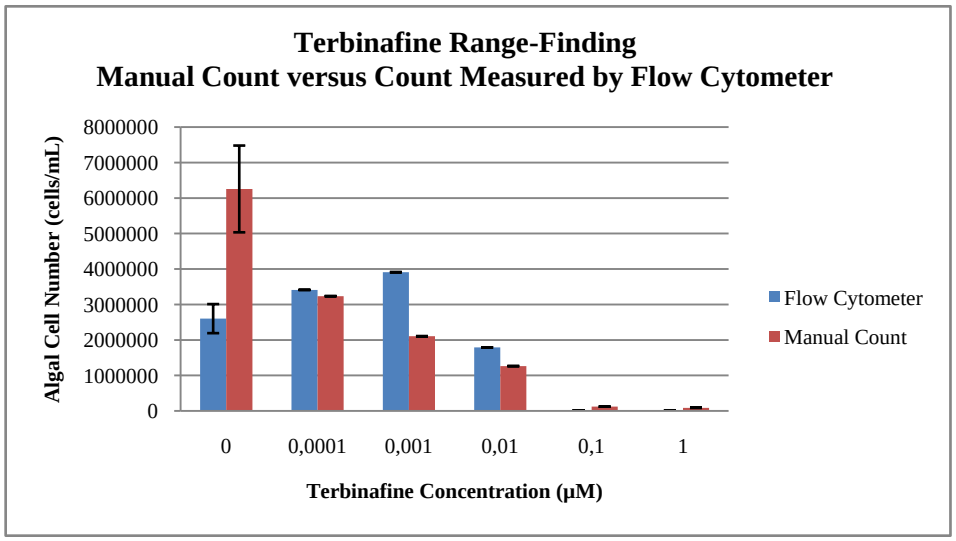

Figure 11. Graph comparing the manual count that was conducted by microscope and the algal cell number, as measured with a flow cytometer after an exposure of 72 hours. Error bars denote standard deviation.

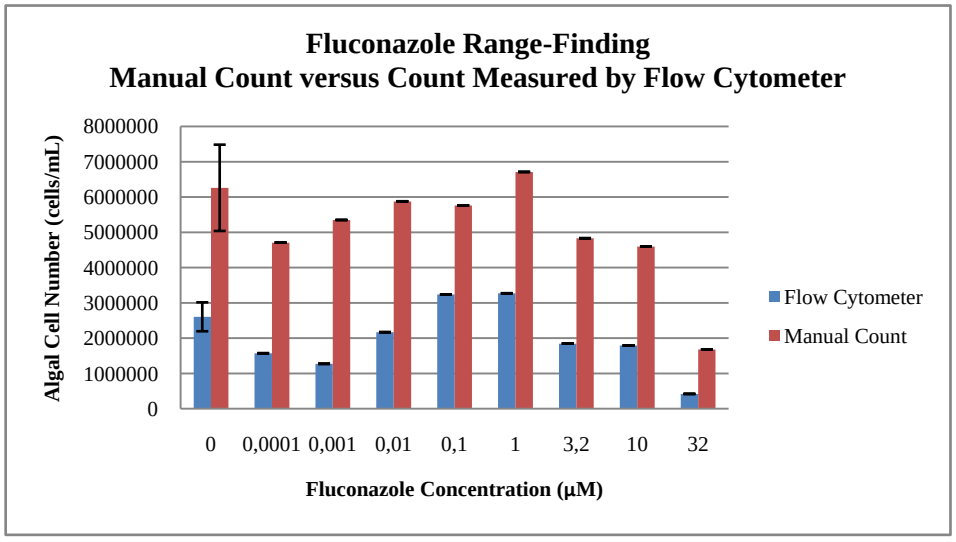

Figure 12. Graph comparing the manual count that was conducted by microscope and the algal cell number, as measured with a flow cytometer after an exposure of 72 hours for the fluconazole treatments. Error bars denote standard deviation.

As the counts measured by the flow cytometer were inaccurate, calculations were not conducted using the daily measurements. However, the inhibition based on biomass and growth rate were calculated using the manual count measurements. As can be seen in Figure 13, an approximately 50% and 100% reduction in biomass was observed at terbinafine concentrations of 0.0001 µM and 0.1 µM, respectively. The inhibition based on growth rate was lower; approximately 60% inhibition of growth rate is observed above 0.1 µM.

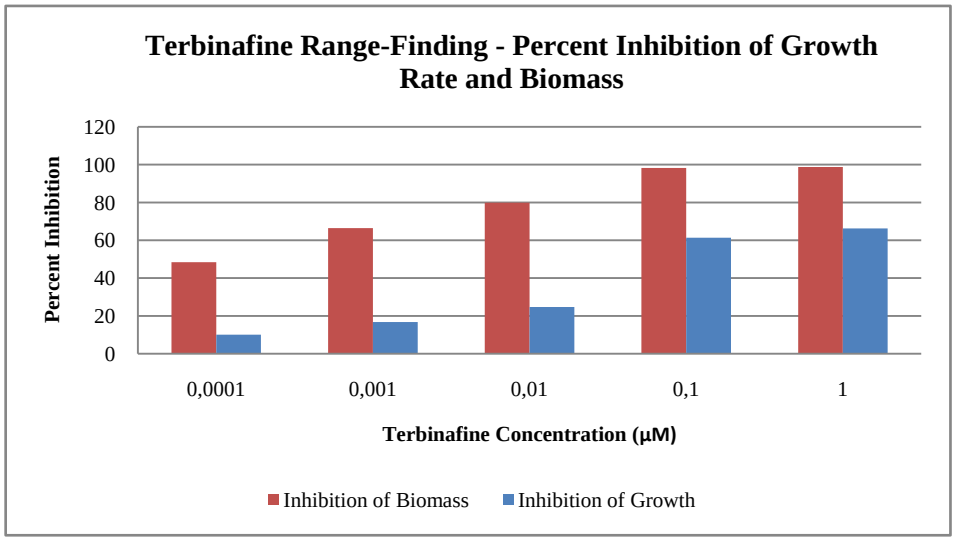

Figure 13. Percent inhibition of biomass and growth rate for the terbinafine treatments, based on a manual cell count after an exposure of 72 hours.

The algae were much more resistant to the fluconazole than to the terbinafine. As can be seen in Figure 14, there is practically zero, or even negative inhibition of the average growth rate, except in the highest treatment

(32 µM). In this treatment, over 70% of the biomass was inhibited and 20% inhibition of average growth rate was measured. It was hypothesized that there was a steep growth inhibition curve between 10 and 32 µM.

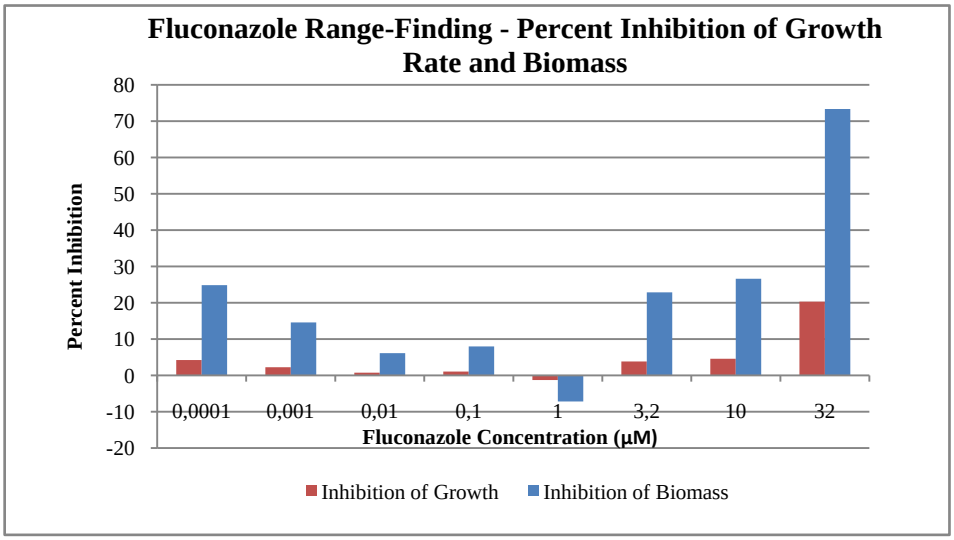

Figure 14. Percent inhibition of biomass and growth rate for the fluconazole treatments, based on a manual cell count after an exposure of 72 hours.

4.1.2 Terbinafine Experiment: December 2009

Seven treatments, ranging from 0.1 to 100 nm were tested, in triplicate, together with three controls. The algal cell number on each day of the experiment is shown in Figure 15.

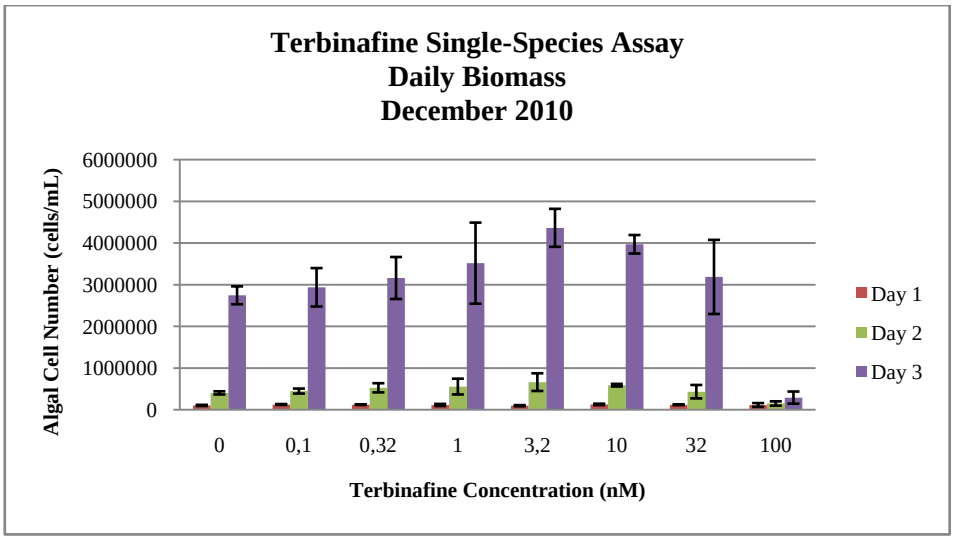

Figure 15. Algal cell number measured on each day of the December 2009 terbinafine experiment. Error bars denote standard deviation.

Terbinafine effects on *P. subcapitata* growth rate are shown in Figure 16 for each treatment. The curve, and corresponding equation, in the figure depicts the growth rate of the controls. On the first day, there is little variation in the growth, with the exception of one of the 100 nM treatments. On the second day, there is high

variation in the growth rate, with the 100 nM treatments having the lowest biomass. On the third day, the 100 nM treatments are well below exponential growth, while the other treatments have similar, or even higher biomass, than the controls.

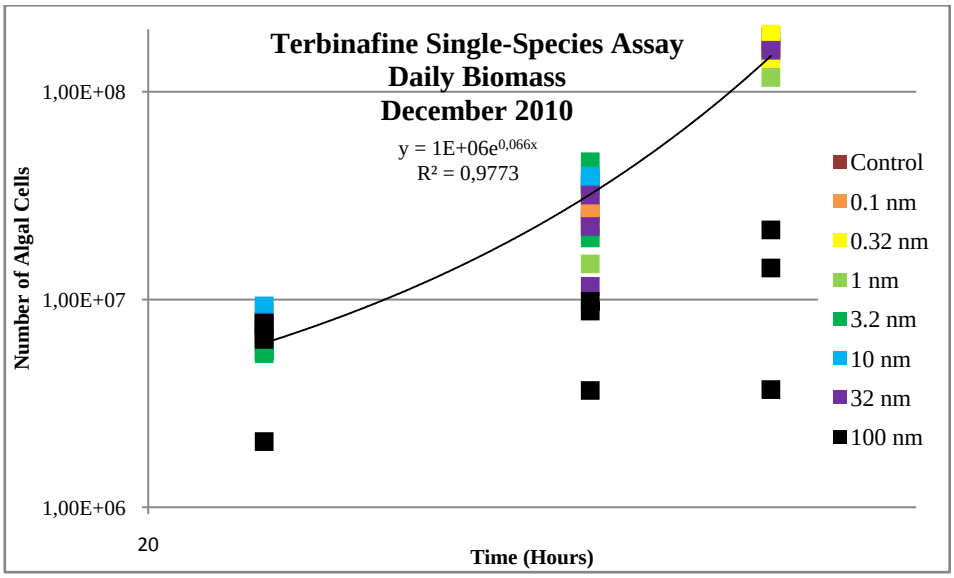

Figure 16. Terbinafine effects on *P. subcapitata* growth. The curve and equation shown is a fit to the controls. The biomass was calculated by multiplying the algal cell number by the volume of fluid in the test tube.

As can be seen in Figure 15 and 16, the growth inhibition curve appears to be steep between 32 and 100 nM. In addition, it is possible that growth stimulation is present at low concentrations (Figure 15). A third terbinafine experiment was necessary, to describe the curve between 32 nM and 100 nM, and also to hopefully decrease the variation in the controls. In this experiment, the percent variation between the controls was approximately 8%, but should be less than 5%.

4.1.3 Control Experiments: January 2010

In an effort to reduce the variation between the controls, the test tubes used for the experiment were replaced. These test tubes were previously used for a nano-particle experiment and it is possible that contamination was still present. In addition, it is possible that algal residue was cross-contaminating experiments. A new washing procedure was started: the test tubes were soaked in a solution of Mucasol® overnight, then scrubbed by hand, then washed in a dishwasher, then rinsed with distilled water and left to air dry.

To determine whether the growth stimulation that was observed was real or an artifact of the experimental setup, five tubes of algae were grown for three days, spaced evenly in the aquaria. The results are shown in Figure 17. The test tubes are numbered according to their location in the aquaria, with number 1 corresponding to the test tube that was located in the far left of the aquaria, and number 5 corresponding to the test tube that was located in the far right of the aquaria. At the end of the experiment, a higher biomass was measured in the middle of the aquaria, which likely indicates that the algae were receiving more light in this location. For all future experiments, the test tubes were rotated daily to decrease the impact of this effect. In addition, any differences in bubbling rate between the test tubes (even though all efforts to avoid this were taken) would hopefully be cancelled out due to the rotation.

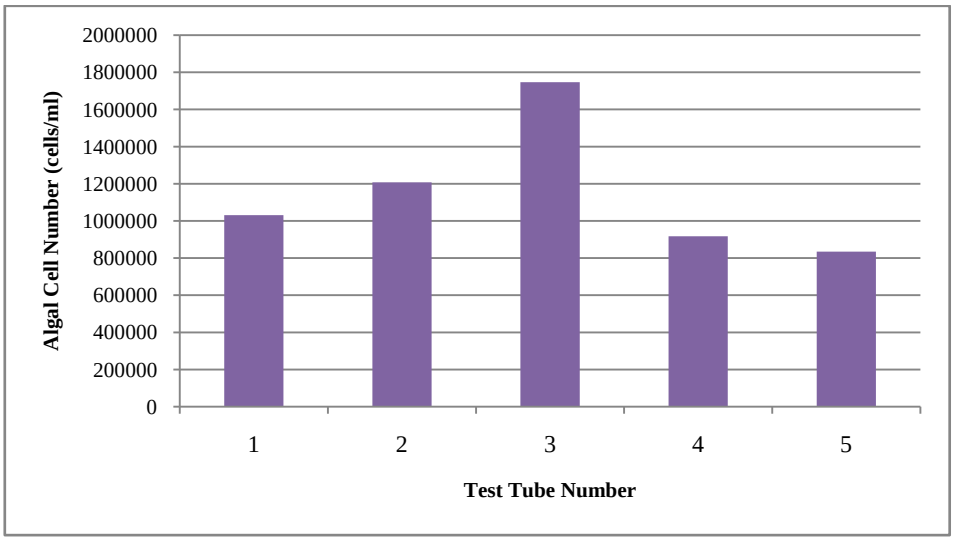

Figure 17. Algal cell number on the final day of a control experiment. The test tubes correspond to position in the aquaria; test tube 1 was located in the far left of the aquaria, and test tube 5 in the far right. There are no replicates.

4.1.4 TERBINAFINE EXPERIMENTS: SPRING 2010

In order to describe the growth inhibition of terbinafine between 10 and 100 nM, another experiment was conducted in March 2010. Seven treatments, ranging from 10 to 100 nm were tested, in triplicate, together with three controls.

The biomass as a function of the log of the time, in hours, is shown in Figure 18 for each treatment. The curve, and corresponding equation, in the figure is a fit to the controls. On the first day, there was little variation in the growth rate between the treatments. On the second day, there was a high variation in the growth rate, with the 100 nm treatments having the lowest biomass. On the third day, the 100 nm treatments were well below exponential growth (Fig. 18). A clear correlation between growth rate and terbinafine concentration was observed (Fig. 18).

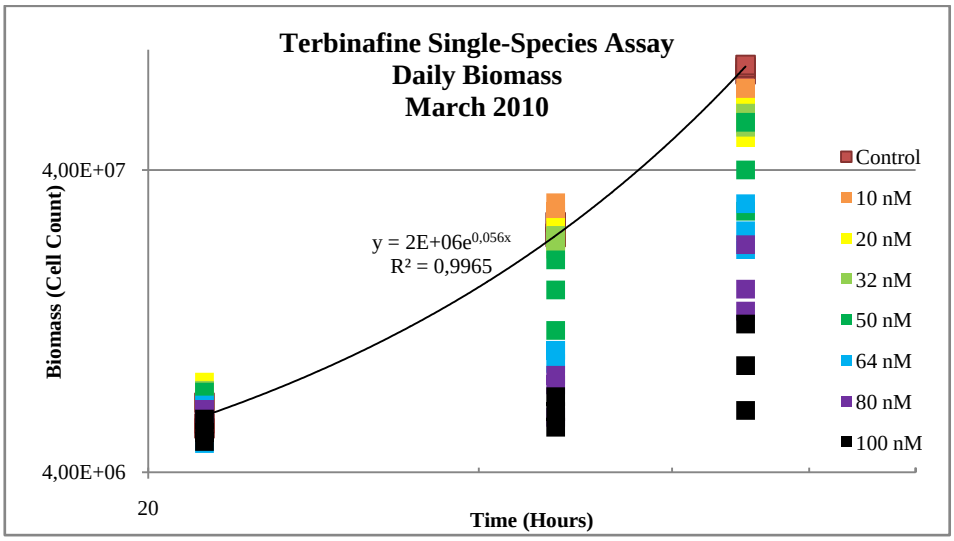

Figure 18. The daily biomass on a log time scale. The curve and equation shown is a fit to the controls. The biomass was calculated by multiplying the algal cell number by the volume of fluid in the test tube.

The average percent inhibition for the terbinafine experiment is shown in Figure 19. The average percent inhibition of biomass over the course of the experiment ranged from approximately 15 percent at 10 nM to approximately 90 percent at 100 nM. The average percent inhibition of growth was lower. Less than 10 percent inhibition of growth was observed in the 10 nM treatments. In the 100 nM treatment, approximately 50 percent inhibition of growth was observed. The average percent inhibition of growth is plotted on a log concentration scale in Figure 20. As can be seen in the figure, the curve is relatively flat in the range of the 10 to 32 nM treatments. The curve begins to increase exponentially started at 50 nM and does not appear to be leveling off at the 100 nM treatment.

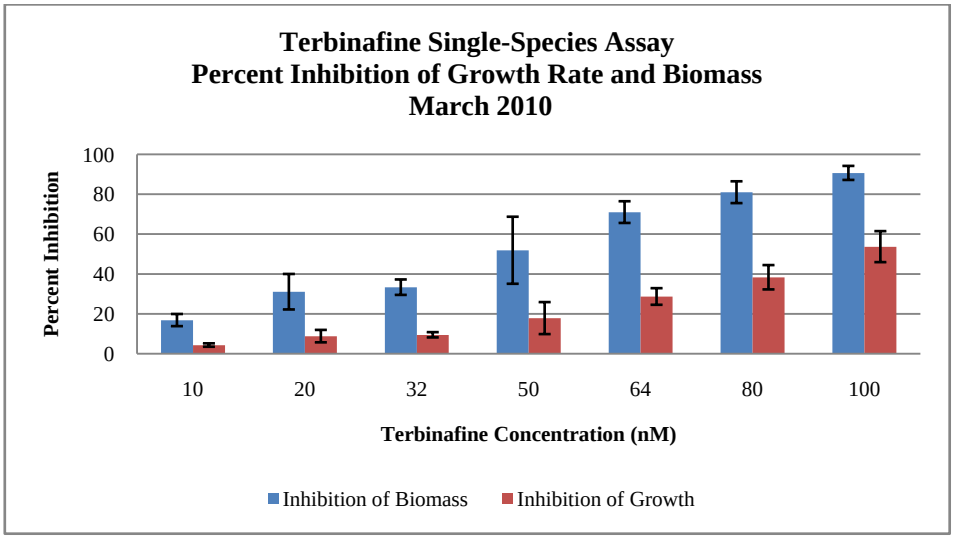

Figure 19. The average percent inhibition of biomass and of growth rate for the experiment. The error bars represent standard deviation.

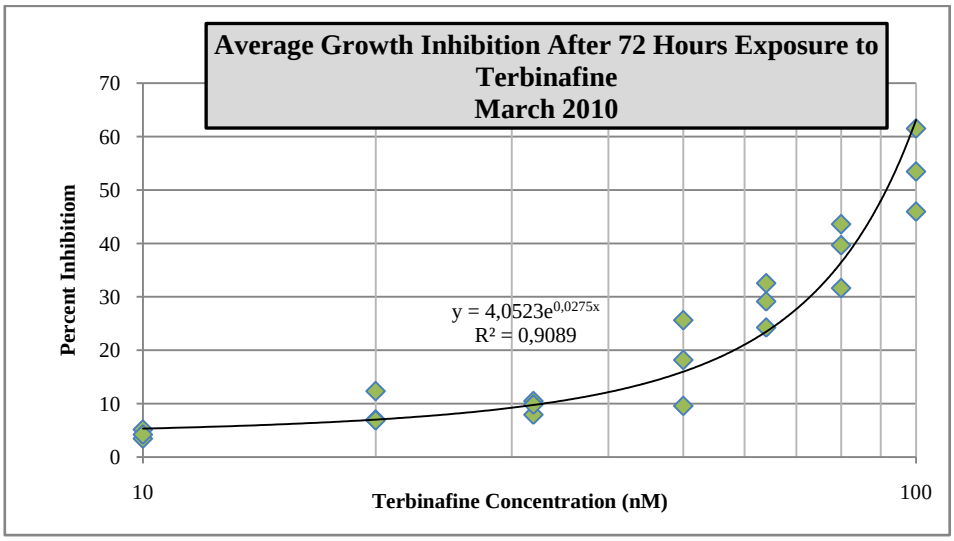

Figure 20. The average percent inhibition of growth rate, plotted on the log of the concentration in each treatment.

4.1.5 Fluconazole Experiments: Spring 2010

Several experiments were conducted with fluconazole in the spring of 2010. Based on the range finding experiment, seven treatments, ranging from 0.5 to 50 µM were tested, in triplicate, together with three controls. After two days, no difference from the controls was observed so the experiment was discarded. The algal cell numbers after two days are shown in Figure 21.

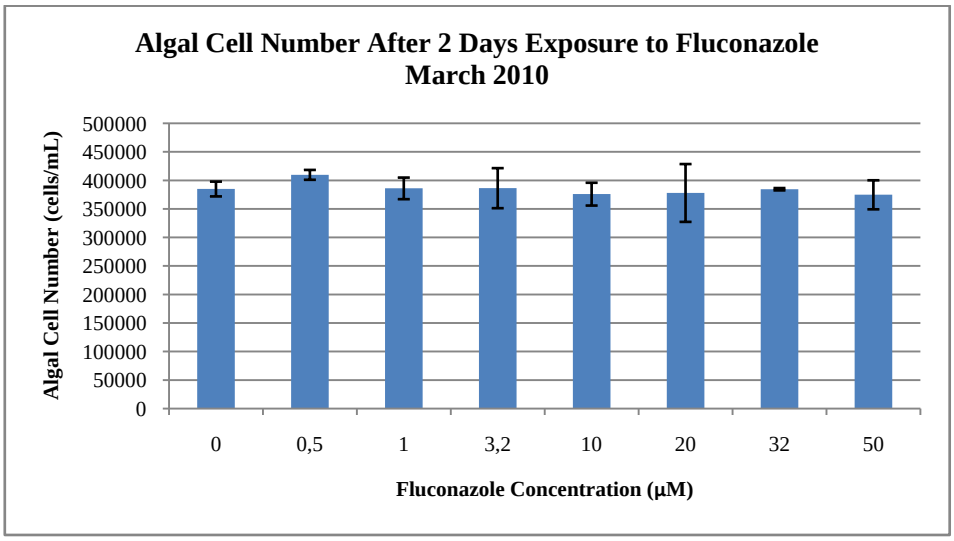

Figure 21. The algal cell numbers for each treatment for a fluconazole experiment conducted in March 2010. The error bars are one standard deviation. As no difference from the controls was observed after two days, the experiment was terminated.

The stock solutions that were used in the experiment were prepared in the fall of 2009. New stock solutions were prepared and the experiment was repeated. The biomass as a function of the log of the time, in hours, is

shown in Figure 22 for each treatment. The curve, and corresponding equation, in the figure is a fit to the controls. On the first day and second days, there was little variation in the growth pattern between the treatments. The treatments were growing at the same rate, or even faster than the controls. On the third day, the 100 µM treatments were deviating from exponential growth, although the difference is not large.

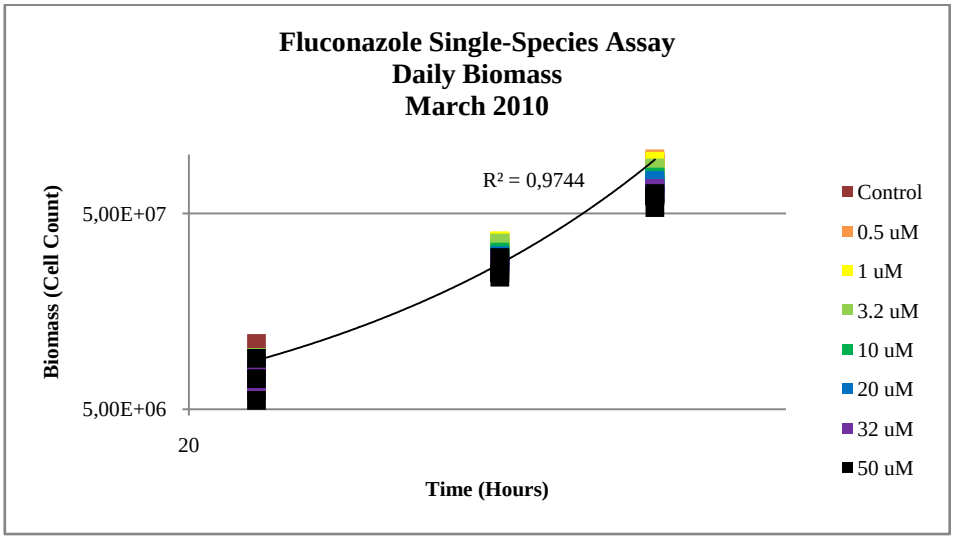

Figure 22. The daily biomass on a log time scale. The curve and equation shown is a fit to the controls. The biomass was calculated by multiplying the algal cell number by the volume of fluid in the test tube.

The average percent inhibition for the fluconazole experiment is shown in Figure 23. A slight stimulation was observed at 0.5 µM. A steady, but shallow increase in both inhibition of growth rate and inhibition of biomass was observed with increasing concentration. At 50 µM, approximately 30 percent inhibition of biomass was observed, while the growth rate was only inhibited by approximately 10 percent. The average percent inhibition of growth is plotted on a log concentration scale in Figure 24. As can be seen in the figure, the curve is very shallow and appears to begin to level off between 32 and 50 µM.

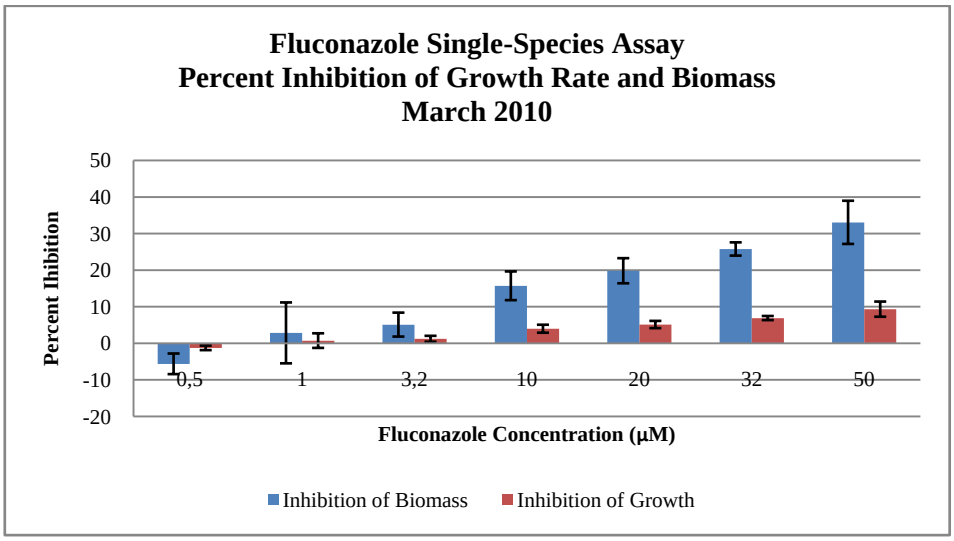

Figure 23. The average percent inhibition of biomass and of growth rate for the experiment. The error bars shown are one standard deviation.

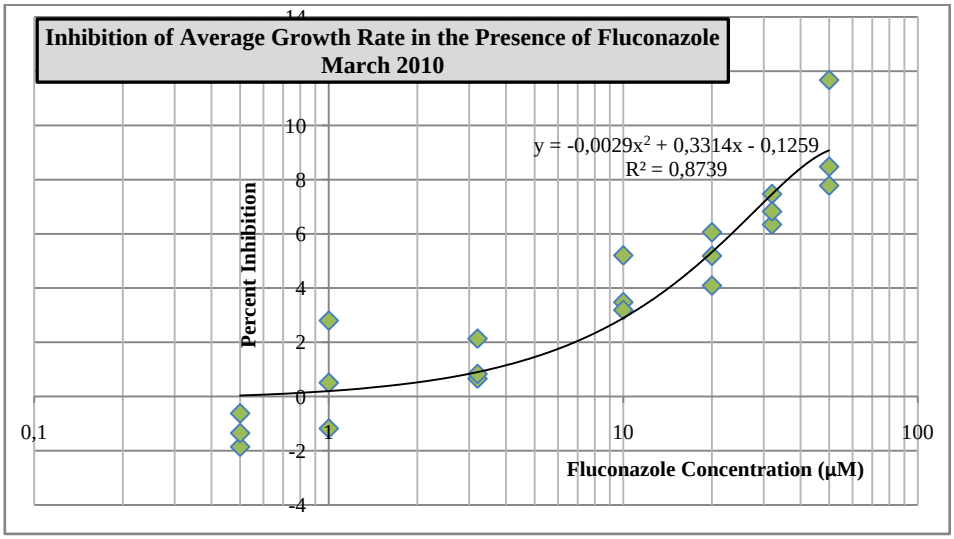

Figure 24. The average percent inhibition of growth rate at the end of the experiment, plotted on the log of the concentration in each treatment.

In order to better describe fluconazole effects at concentrations ranging from 10 to 100 µM, an additional experiment was conducted in April 2010. Seven treatments, ranging from 10 to 100 µM were tested in triplicate, together with three controls. The biomass as a function of the log of the time, in hours, is shown in Figure 25 for each treatment. The curve, and corresponding equation, in the figure is a fit to the controls. On the first day, there was little variation in the growth pattern between the treatments. On the second day, there again was little variation. The treatments were growing at the same rate, or even faster than the controls. On the third day, an increasing deviation from exponential growth was observed, beginning with the 64 µM treatment. The largest deviation from exponential growth was observed in the 100 µM treatment.

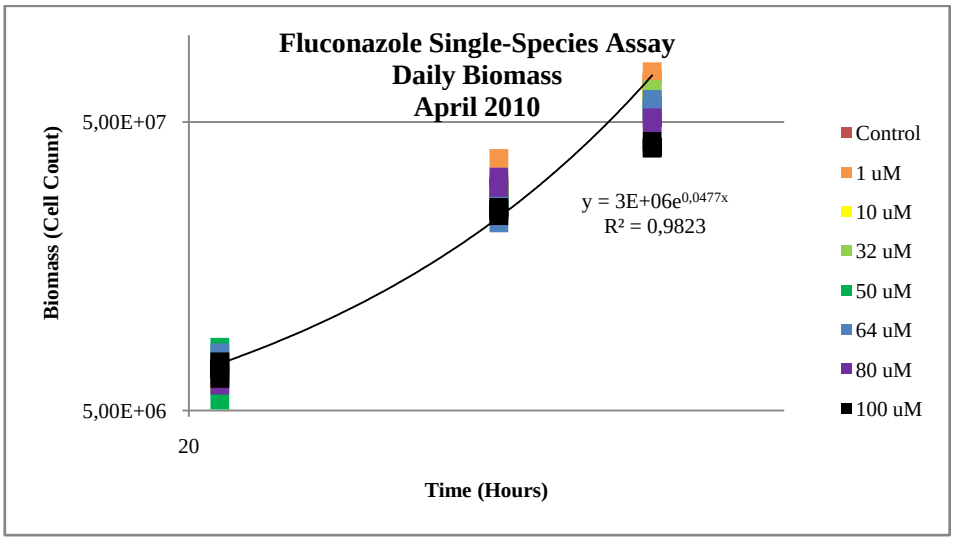

Figure 25. The daily biomass on a log time scale. The curve and equation shown is a fit to the controls. The biomass was calculated by multiplying the algal cell number by the volume of fluid in the test tube.

The average percent inhibition for the April 2010 fluconazole experiment is shown in Figure 26. A slight stimulation was observed at 1 µM. A steady, but shallow increase in both inhibition of growth rate and inhibition of biomass was observed with increasing concentration. At 100 µM, approximately 35 percent inhibition of biomass was observed, while the growth rate was only inhibited by approximately 10 percent. The average percent inhibition of growth is plotted on a log concentration scale in Figure 27. As can be seen in the figure, the curve is very shallow but appears to begin increasing exponentially at 50 µM.

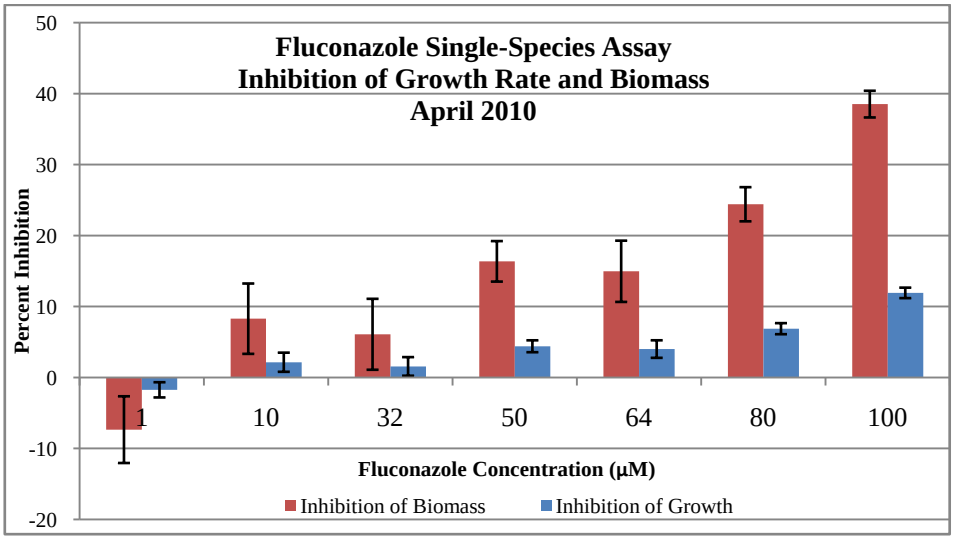

Figure 26. The average percent inhibition of biomass and of growth rate for the experiment. The error bars shown are one standard deviation.

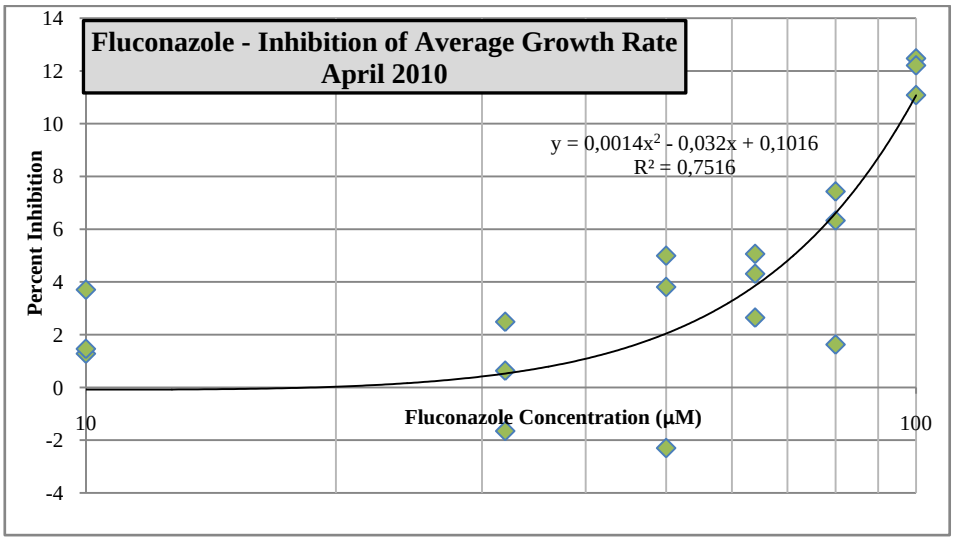

Figure 27. The average percent inhibition of growth rate at the end of the experiment, plotted on the log of the concentration in each treatment.

4.2 Single-Species Assays – Pigment Measurements

4.2.1 Effects on Pigments - Terbinafine

At the end of the last terbinafine experiment, in March 2010, pigment samples were taken and analyzed using HPLC. The sum of chlorophyll *a* and its degradation products (chlorophyll *a*), are shown in Figure 28, which gives an indication of biomass. Overall, there is a trend that the content of chlorophyll *a* decreased, as the concentration of terbinafine increased. The data did exhibit quite a large variation between the replicates. The coefficient of variation was 21%. The violaxanthin content, divided by the sum of violaxanthin, antheraxanthin, and zeaxanthin (V/VAZ) is shown in Figure 29. The V/VAZ content is a measure of oxidative stress in green algae.[16] There was much less variation in the V/VAZ content. The standard deviation divided by the mean for the controls was 0.018. The V/VAZ ratio showed little variation between the controls and all the treatments, up to 64 µM. The ratio was approximately 0.8 for these samples. The V/VAZ ratio for the 80 µM sample was 0.76, and for the 100 µM sample, was 0.6. With a 95% confidence interval, a statistically significant decrease was observed at 100 µM.

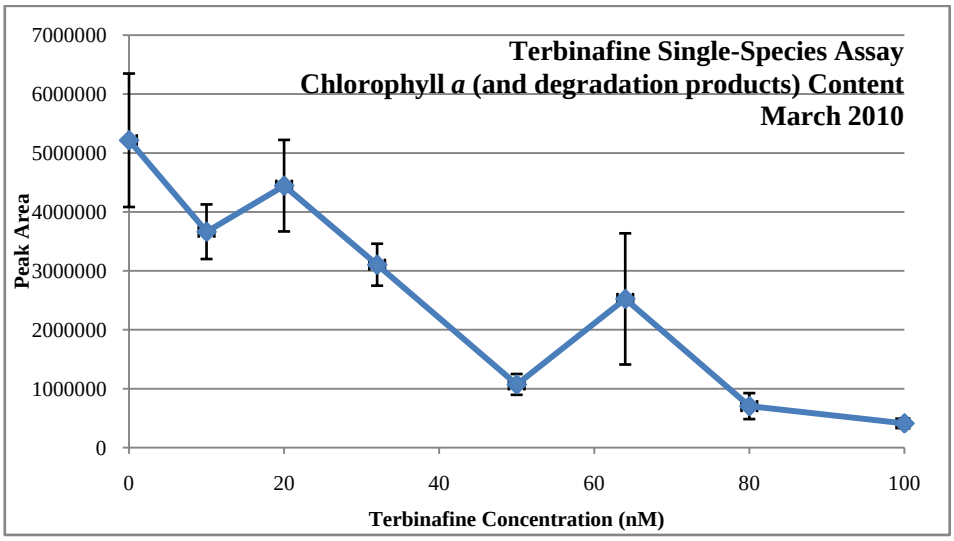

Figure 28. The sum of the chlorophyll *a* content, and its degradation products for the March 2010 terbinafine experiment. Pigments were analyzed using HPLC. There are three replicates for each data point, and the error bars represent standard deviation.

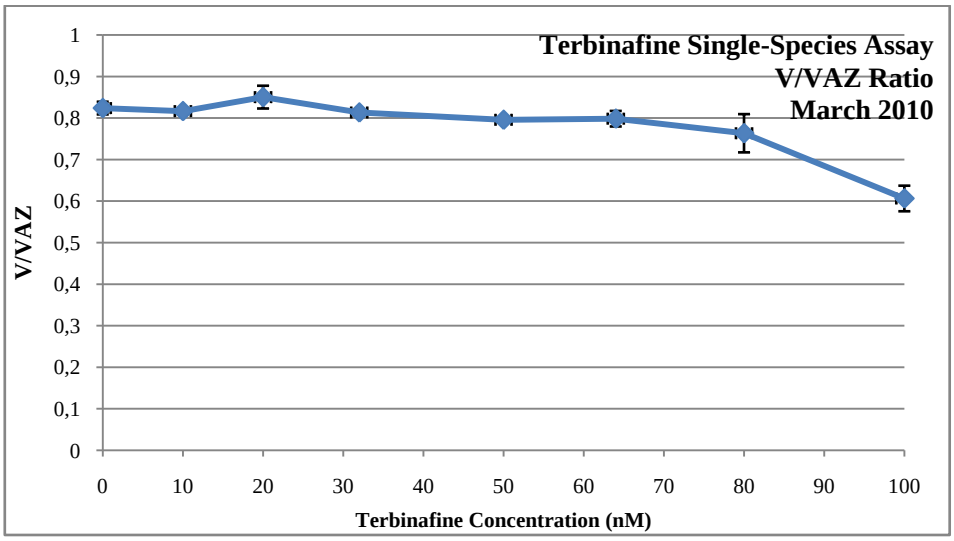

Figure 29. The violaxanthin content, divided by the sum of the peak areas for violaxanthin, antheraxanthin, and zeaxanthin. Samples were taken on the last day of the March 2010 terbinafine experiment. The error bars represent standard deviation.

4.2.2 Effects on Pigments - Fluconazole

At the end of the last fluconazole experiment, in April 2010, pigment samples were taken and analyzed using HPLC. The sum of chlorophyll *a* and its degradation products (chlorophyll *a*), are shown in Figure 30. Similar to the chlorophyll *a* content measured after the terbinafine experiment, there was quite a large variation between the replicates. The standard deviation divided by the mean for the controls was 0.22. The chlorophyll *a* content decreased with increasing concentration, to 50% of the control level at 64 µM. Above 80 µM, the chlorophyll *a* content was higher than at 64 µM; however, there was considerable variation

between the replicates for this treatment. At 100 µM, the chlorophyll *a* content was above that of the controls. The violaxanthin content is also shown in Figure 30, and a similar trend was observed. The violaxanthin content decreased with increasing terbinafine concentration, to approximately 40% of the control level at 64 µM. Similar to chlorophyll *a*, an increasing trend was observed in the 80 and 100 µM treatments. A similar trend was observed with antheraxanthin and zeaxanthin (data not shown), but at lower concentrations than Chlorophyll *a* and violaxanthin. The V/VAZ ratio is shown in Figure 31. There was almost no variation in the V/VAZ content, both between replicates, and between treatments. The standard deviation divided by the mean for the controls was 0.021. The V/VAZ ratio was approximately 0.83 in the controls, and was 0.785 in the highest treatment, 100 µM. A statistically significant change in the V/VAZ ratio was not observed.

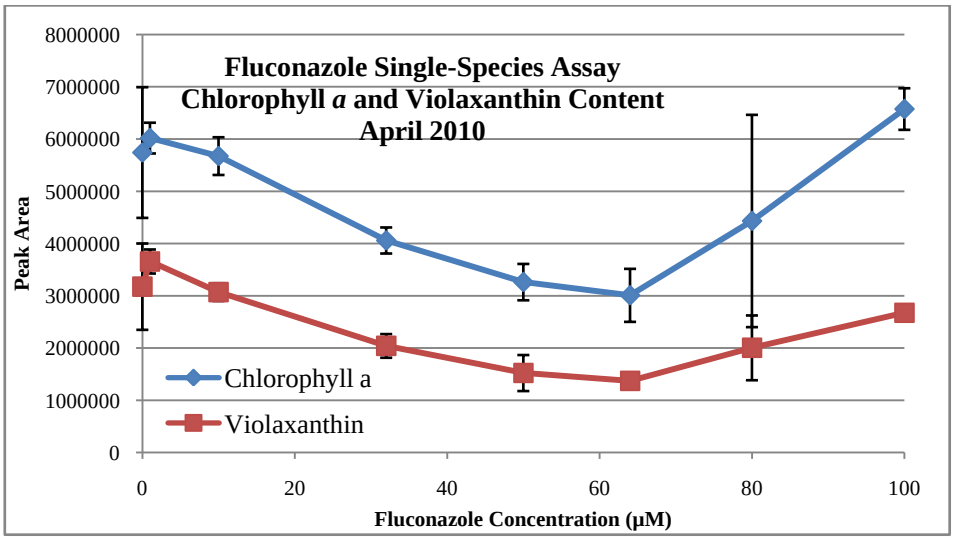

Figure 30. The chlorophyll *a* (and its degradation products) and the violaxanthin content for the April 2010 fluconazole experiment. Pigments were analyzed using HPLC. The error bars represent standard deviation.

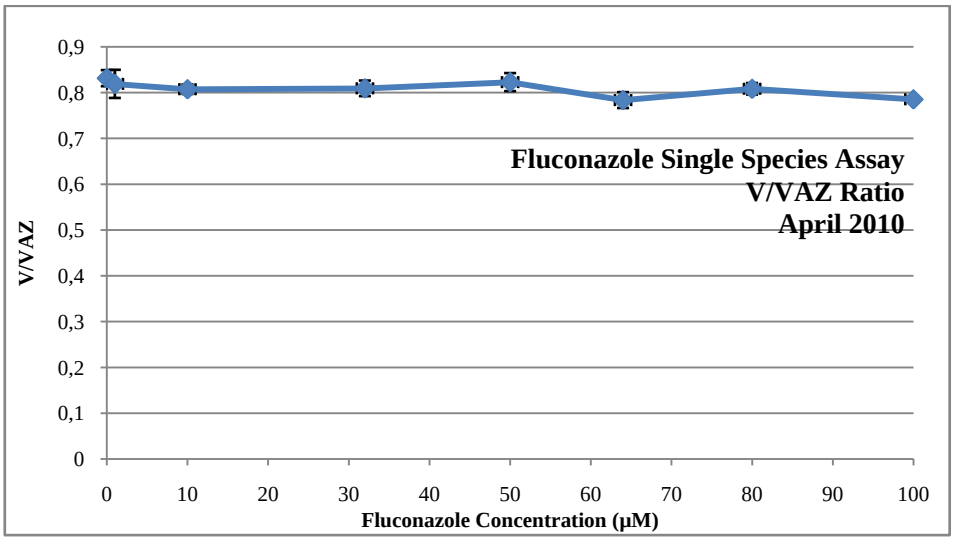

Figure 31. The violaxanthin content, divided by the sum of the peak areas for violaxanthin, antheraxanthin, and zeaxanthin. Samples were taken on the last day of the April 2010 fluconazole experiment. The error bars represent standard deviation.

4.2 Single-Species Assays – Sterol Measurements

4.2.1 Effects on Sterols - Terbinafine

Sterols were extracted after the last terbinafine experiment, in March 2010, as described in Section 3.3. The peak areas were normalized to the internal standard and weighted for the biomass of the sample. Six end-product sterols were identified in the controls. One of the chromatograms from the control samples is shown in Figure 32. All of these sterols are phytosterols: Δ8,22-ergastadienol, Δ7,22-ergastadienol, ergostenol, chondrillasterol, 24,5α-cholesta-5,7,22-trien-3β-ol, and Δ7-chondrillastenol. The percentage of each sterol in the controls is shown in Table 2. The dose-response curves for each of these sterols are shown in Figure 33.

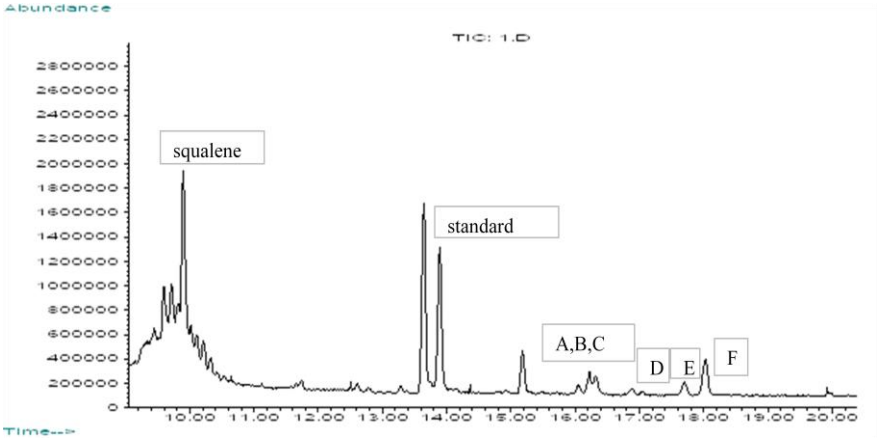

Figure 32. Chromatogram from one of the controls associated with the fluconazole experiment. The standard used was cholestanol. The sterols identified are listed in Table 2.

Table 2 Sterol Content of the Controls at the End of the March 2010 Terbinafine Experiment	
Sterol Name	*Percent of the Total Sterol Composition*
Δ8,22-ergastadienol (A)	10%
Δ7,22-ergastadienol (B)	17%
Ergostenol (C)	7%
Chondrillasterol (D)	7%
24-ethyl-5α-cholesta-5,7,22-trien-3β-ol (E)	12%
Δ7-chondrillastenol (F)	46%

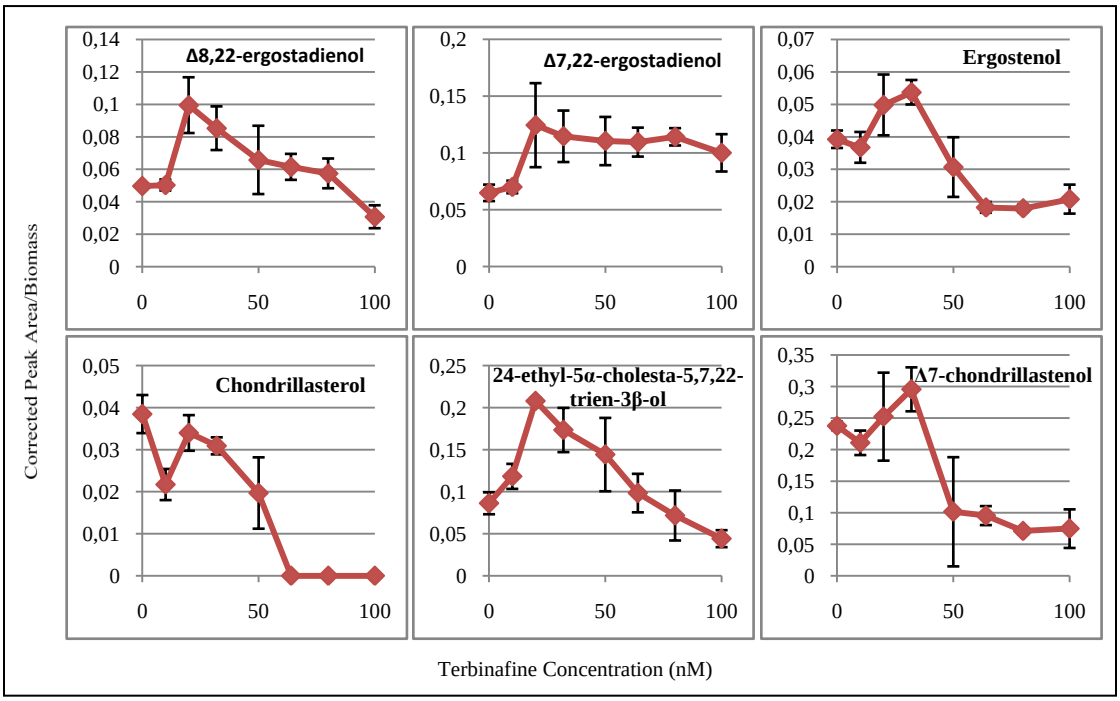

Figure 33. The dose response curves to terbinafine for the sterols in *P. subcapitata* after 72-hours of exposure. For the controls, there were two replicates. There were three replicates for all of the treatments. The error bars represent standard deviation.

For all of the end-product sterols, with the exception of chondrillasterol, there appeared to be a stimulation at low concentrations. The concentration of Δ7,22-ergastadienol actually increased as terbinafine concentration increased. Similarly, the concentration of Δ8,22-ergastadienol was above control levels for the majority of the treatments; it is only the 100 nM treatment where the concentration was below control levels. The concentration of ergostenol, 24,5α-cholesta-5,7,22-trien-3β-ol, and Δ7-chondrillastenol decreased to at least half of the control content as the concentration of terbinafine increased to 100 nM. Chondrillasterol was not detected in the 64, 80 and 100 nM treatments.

As the concentration of terbinafine increased, the total sterol content also decreased, with the exception of the 10 and 20 nM treatments, for which there was a stimulation of sterol content, as shown in Figure 33.

The end-product sterols, and squalene, as a fraction of the sum of the sterol and squalene content, are shown in Figure 34. As can be seen in the figure, the fraction of squalene increased as the concentration of terbinafine increased. Squalene was 20% of the total at 50 nM, and increased to approximately 85% of the total, at 100 nM.

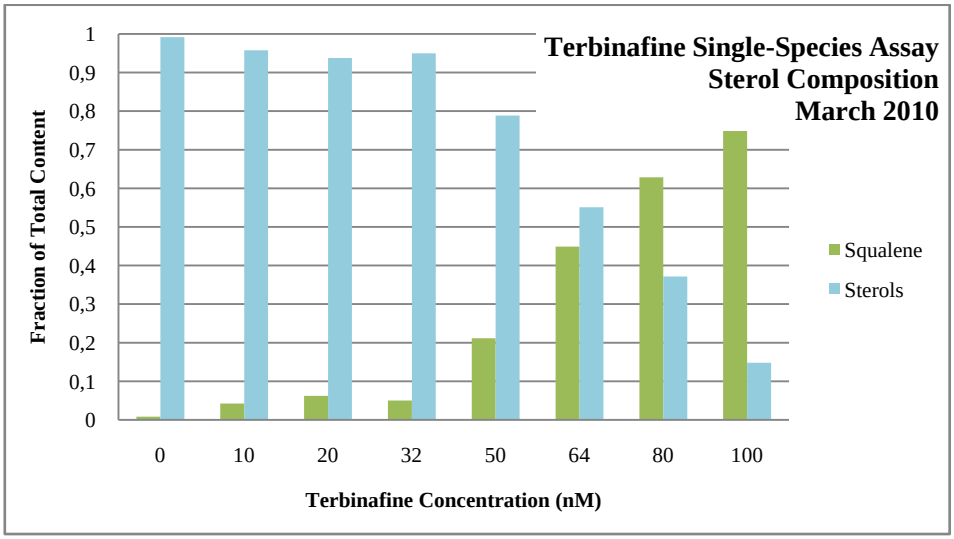

Figure 34. The percentage of end product sterols and squalene, as a fraction of the sum of the sterol and squalene content, at the end of the 72-hour treatment. Each bar is an average of three samples, with the exception of the control, which is an average of two samples.

4.2.2 Effects on Sterols - Fluconazole

Sterols were extracted after the last fluconazole experiment, in April 2010, as described in Section 3.3. The peak areas were normalized to the internal standard and weighted for biomass. The same six end-product sterols which were identified in the terbinafine controls, were also identified in the controls for the fluconazole experiment. The percentage of each sterol in the control sample is shown in Table 3. As the fluconazole concentration increased, the concentration of each of these sterols decreased, as shown in Figure 35. At 32 μM, the concentration of all six of the sterols was below half that of control levels. For the majority of the sterols, the dose-response curve was flat above 50 μM. The only exception was chondrillasterol. The concentration of chondrillasterol actually increased above 64 μM; however, even at 100 μM, the concentration was still less than half of the control levels.

Table 3 Sterol Content of the Controls at the End of the April 2010 Fluconazole Experiment	
Sterol Name	*Percent of the Total Sterol Composition*
Δ8,22-ergastadienol	15%
Δ7,22-ergastadienol	21%
Ergostenol	4%
Chondrillasterol	5%
24-ethyl-5α-cholesta-5,7,22-trien-3β-ol	24%
Δ7-chondrillastenol	30%

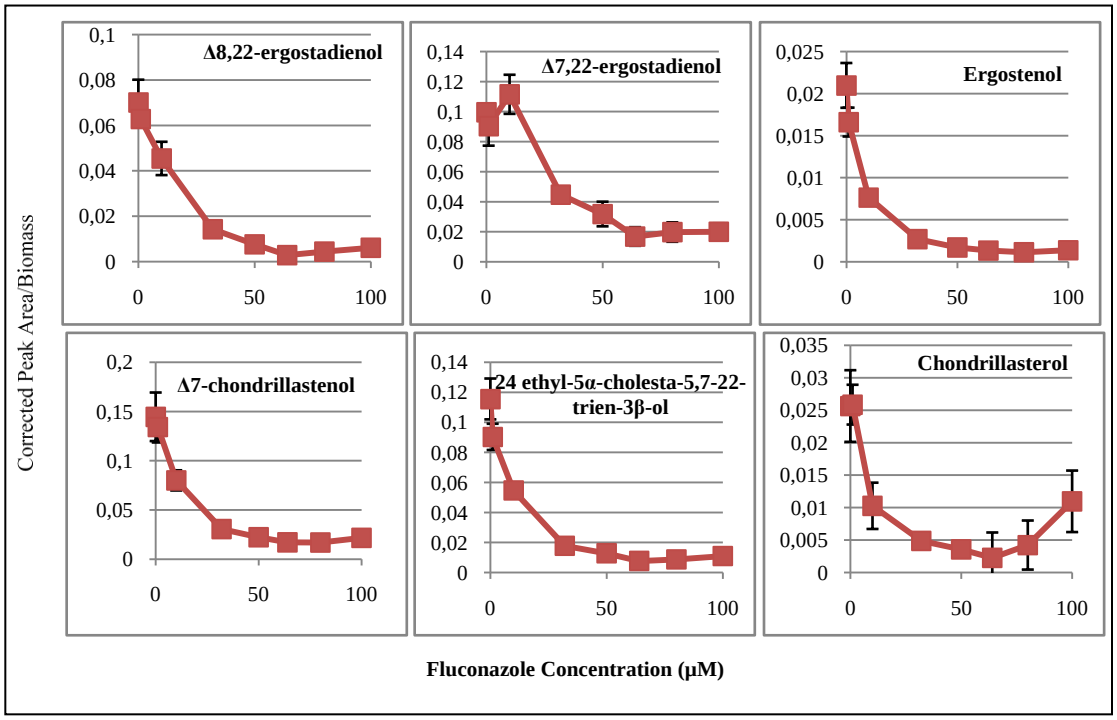

Figure 35. The dose response curves to fluconazole for the sterols in *P. subcapitata* after 72-hours of exposure. The error bars represent standard deviation (n=3).

As the concentration of fluconazole increased in the treatments, the concentration of four 14α-methylated sterols, which were not present in the controls, increased. The dose-response curves for these sterol intermediates are shown in Figure 36.

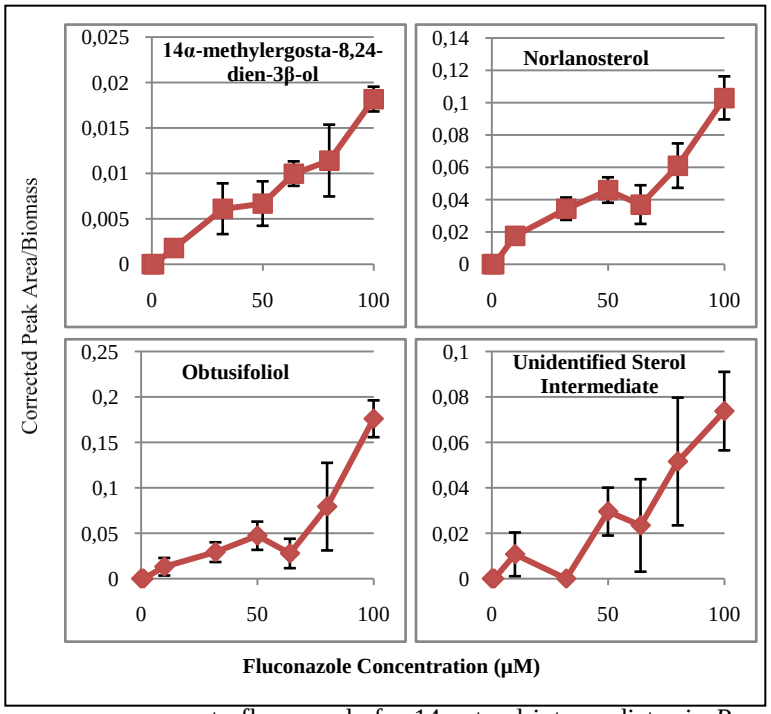

Figure 36. The dose response curves to fluconazole for 14α-sterol intermediates in *P. subcapitata* after 72-hours of exposure. For the controls, and all of the treatments, there were three replicates. The error bars represent one standard deviation.

The end-product sterols, and the 14α-sterol intermediates, as a fraction of the sum of both, are shown in Figure 37. As can be seen in the figure, the fraction of 14α-sterol intermediates increased as the concentration of fluconazole increased. 14α-sterol intermediates were present at a concentration 10 µM, and increased to 80% of the total sterol content, at 100 µM.

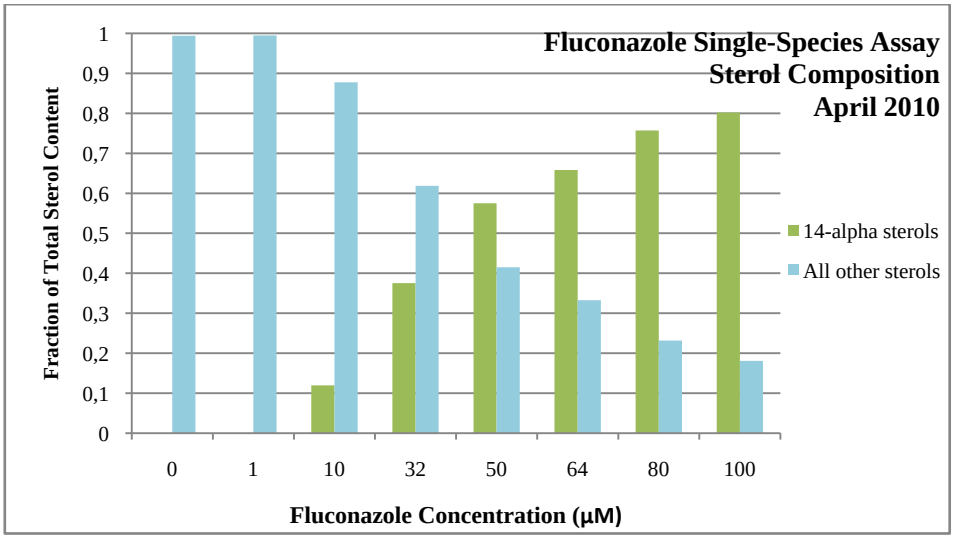

Figure 37. The fraction of sterols and 14-α sterol intermediates, in relation to the sum of both, at the end of the 72-hour treatment. Each bar is an average of three replicates.

The concentration of squalene also increased as the concentration of fluconazole increased, as shown in Figure 38. However, this only occurred at a fluconazole concentration of 100 µM. The relative content of squalene in the 100 µM treatment was approximately 2%.

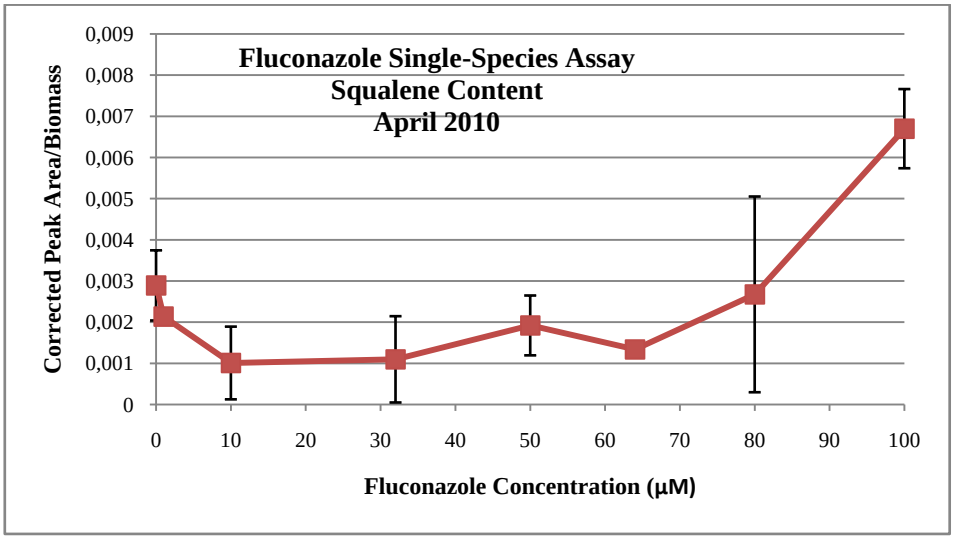

Figure 38. The squalene content for each treatment after 72-hours of exposure. Each data point represents the average of three replicates. The error bars represent standard deviation.

4.3 SWIFT Assay – Pigment Measurements

4.3.1 Effects on Periphyton Pigments in the SWIFT Test - Terbinafine

Terbinafine effects on periphyton chlorophyll *a* content is shown in Figure 39. As can be seen in Figure 39, the 10, 20 and 32 nM samples appeared to have chlorophyll *a* content above that of the controls, though it is not statistically significant. The 50, 64, 80 and 100 nM samples had control levels of chlorophyll *a*.

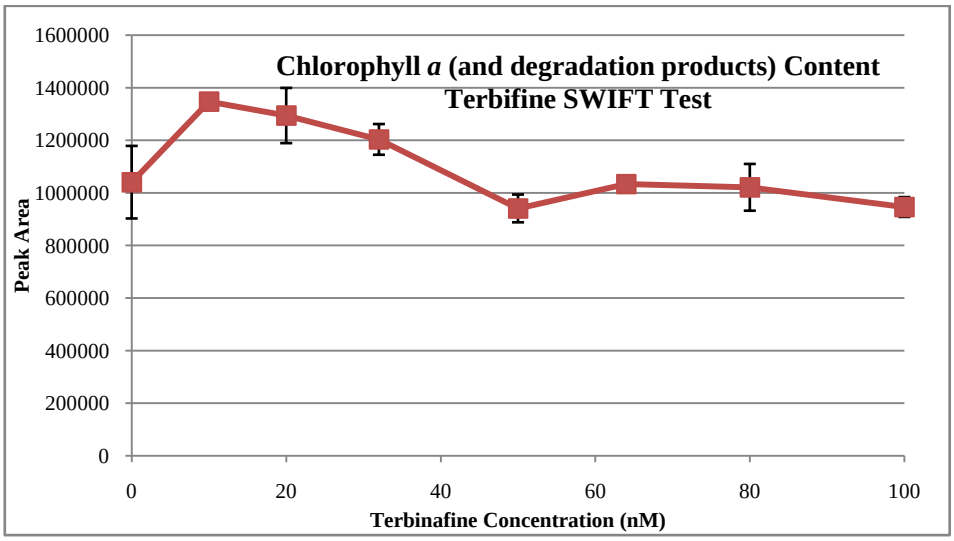

Figure 39. The sum of the chlorophyll *a* content, and its degradation products for the SWIFT terbinafine experiment. Pigments were analyzed using HPLC. For the controls and the 10 nM data points, there were two replicates. For the 64 nM data point, there was only one replicate. For the remainder of the data points, there were three replicates. The error bars represent one standard deviation.

Terbinafine effects on lutein, which is a pigment specific to green algae, and the fluoxanthin content, which is a pigment specific to diatoms, is shown in Figure 40. A clear reduction in lutein content was observed already at low terbinafine concentrations. At 20 nM, the lutein content was reduced by 50%, and this decrease continues at higher concentrations. Above 64 nM, the lutein content was relatively constant, at 20% of the control level (Fig. 40).

The fluoxanthin content appeared to be stimulated compared to control levels, in the 10, 20 and 32 nM treatments. At 50 nM and above, the fluoxanthin level was comparable to control levels (Fig. 40).

In Figure 41, the V/VAZ ratio and the ratio the diadanoxanthin content, divided by the sum of the peak areas for diadanoxanthin and diatoxanthin (DD/DD+DT) are shown. The V/VAZ content is a measure of oxidative stress in green algae.[16] The DD/DD+DT content is a measure of oxidative stress in diatoms.[16] The V/VAZ content decreased from 0.8 to 0.6 as the concentration of terbinafine increased. The DD/DD+DT content decreased from 0.9 to 0.8 as the concentration increased. There was also a higher standard deviation between the replicates with increasing concentration.

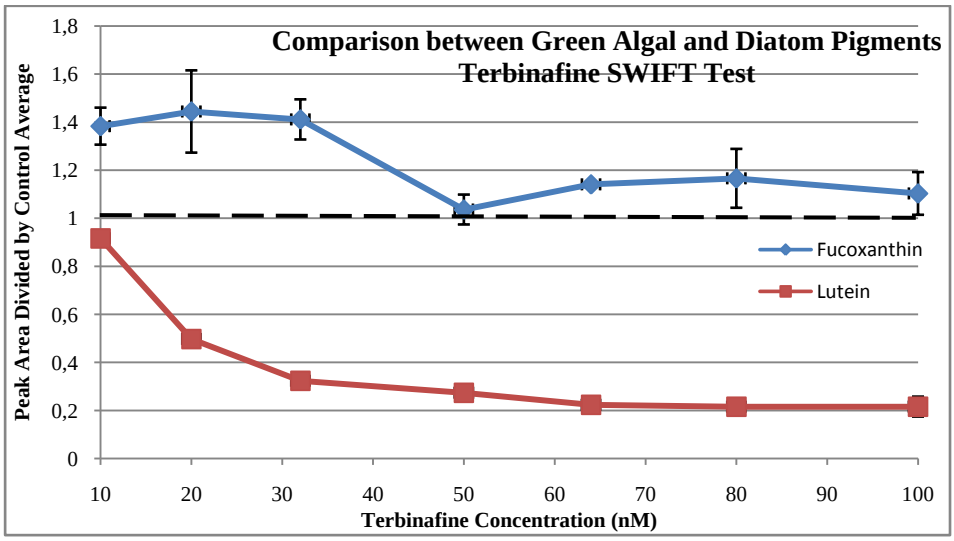

Figure 40. The lutein and the fucoxanthin content in each of the terbinafine treatments at the end of the SWIFT experiment. The pigment content in each replicate has been divided by the control average. There were three replicates for each data point, with the exception of the 10 nM treatment, for which there were two replicates, and the 64 nM treatment, for which there was only one replicate. The error bars denote standard deviation.

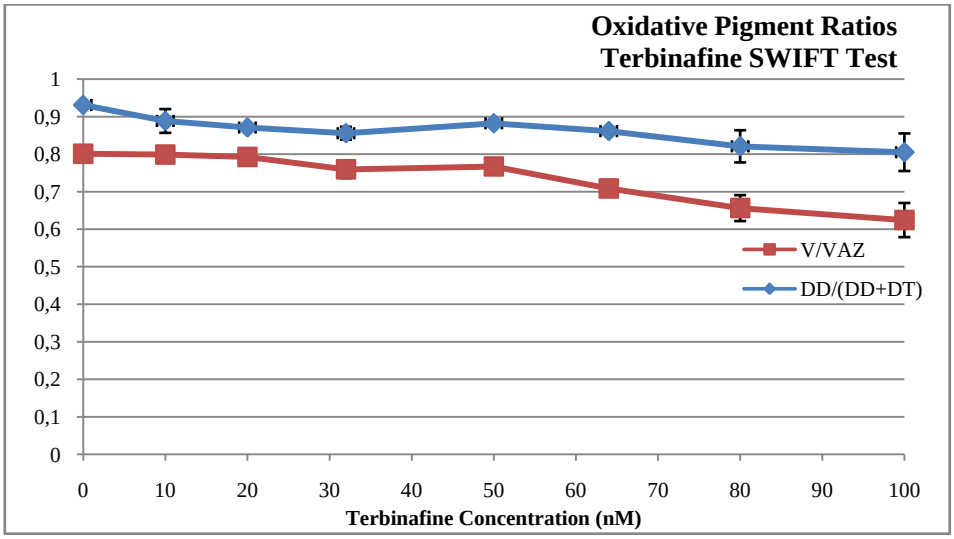

Figure 41. The xanthophyll pigment ratios in each of the terbinafine treatments at the end of the SWIFT experiment. The red line is the violaxanthin content, divided by the sum of the peak areas for violaxanthin, antheraxanthin, and zeaxanthin (V/VAZ). The blue line is the diadanoxanthin content, divided by the sum of the peak areas for diadanoxanthin and diatoxanthin (DD/(DD+DT)). There were three replicates for each data point, with the exception of the 10 nM treatment, for which there were two replicates, and the 64 nM treatment, for which there was only one replicate. The error bars denote standard deviation.

4.3.2 Effects on Periphyton Pigments in the SWIFT Test - Fluconazole

The chlorophyll *a* content measured from the fluconazole treatments is shown in Figure 42. There were three controls, and one replicate for each of the treatments. Without replicates, it is difficult to determine whether

there is a trend in the data. The 80 µM data point appears lower than control levels, but it is difficult to tell whether there really was an effect. We did not visually observe a difference between the discs at the end of the experiment; this observation correlates well with the chlorophyll *a* results.

Fucoxanthin followed a similar trend to the chlorophyll *a* content, as shown in Figure 43. The lutein content was higher than the control average for the 10 and 32 µM treatments, after which the trend was similar to the flucoxanthin pigment. Again, it was difficult to determine whether a trend is really present due to the lack of replicates.

The V/VAZ and the DD/DD+DT content for the fluconazole treatments are shown in Figure 44. The two ratios follow nearly an identical path. There was little variation as the fluconazole concentration increased, indicating that there was little oxidative stress or direct effects on photosynthesis.

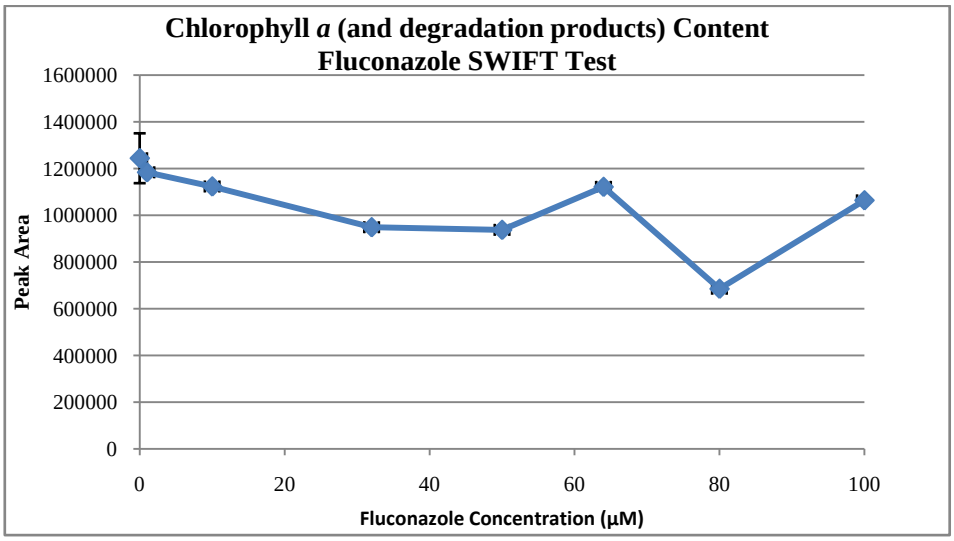

Figure 42. The sum of the chlorophyll *a* content, and its degradation products for the SWIFT fluconazole experiment. Pigments were analyzed using HPLC. For the controls, there were three replicates. For the treatments, there was one replicate. The error bars denote standard deviation.

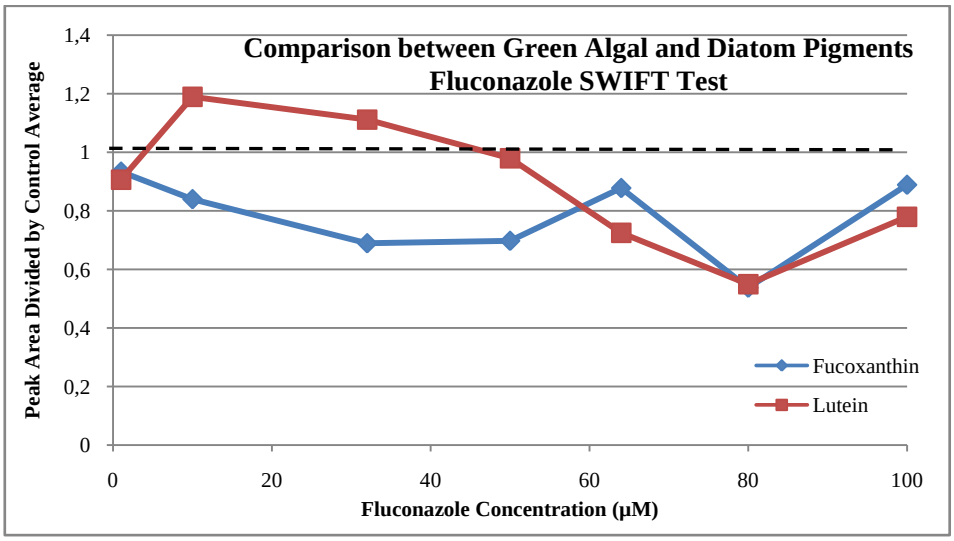

Figure 43. The lutein and the fucoxanthin content in fluconazole treatments at the end of the SWIFT experiment. The pigment content in each treatment has been divided by the control average. There was only one replicate for each data point.

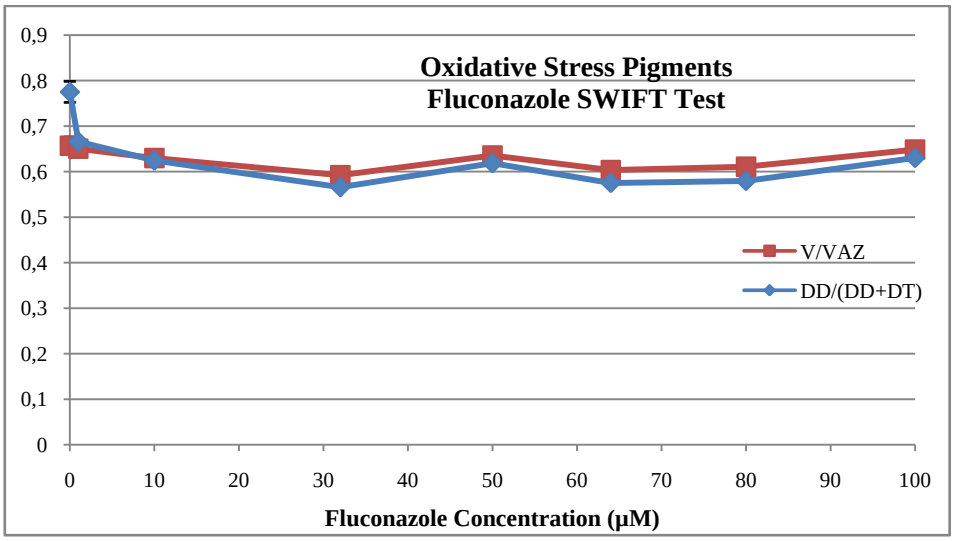

Figure 44. The xanthophyll pigment ratios in each of the terbinafine treatments at the end of the SWIFT experiment. The red line is the violaxanthin content, divided by the sum of the peak areas for violaxanthin, antheraxanthin, and zeaxanthin (V/VAZ). The blue line is the diadanoxanthin content, divided by the sum of the peak areas for diadanoxanthin and diatoxanthin (DD/(DD+DT)). There were three controls. For each treatment there was only one replicate. The error bars denote standard deviation.

5.0 DISCUSSION

A substantial amount of time during this project was spent on method development. Experiments run in the fall of 2009 were discarded either due to inaccuracies associated with cell counting or because the percent difference between the controls was too high (greater than 10%). Several adjustments were made to improve the system. First, it was recognized immediately that the flow rate in the flow cytometer (even with weighing samples before and after each run) was too unstable to determine an algal cell number just based on flow rate. An absolute count standard was used to calibrate fluorescent micro-beads, which were subsequently added to each sample. As can be seen in Figure 10 (in the Methods section), this substantially improved the accuracy and precision of the flow cytometer.

Several changes were also made to the setup of the single-species assay, in an effort to reduce the percent variation between the controls. The test-tubes used in the experiments in the fall had previously been used for a nano-particle experiment. Since it was possible that the test-tubes could still be contaminated, a new set of test-tubes was purchased. In addition, soap had previously been used for washing. A new washing procedure was started. The test-tubes were first soaked overnight in Mucasol®, which is a laboratory detergent, then scrubbed by hand. Next, the test-tubes were washed in a dishwater, then rinsed with distilled water prior to autoclaving. In addition, every effort was made to ensure that the air supply to each of the test-tubes was equal. This had to be done by eye, and it is difficult to make fine-tune adjustments with the current setup, which uses plastic stop-cocks to regulate the air flow. It is recommended that new stop-cocks be purchased, which are easier to adjust. Lastly, a higher growth rate was measured in the middle of the aquaria, as shown in Figure 17 (in the Results section). Subsequently, the test-tubes were rotated daily, so that over the course of the 72-hour treatment, each test-tube was located on the left side of the aquaria for 24 hours, in the middle for 24-hours, and on the right side of the aquaria for 24 hours. In addition to evening out the light exposure, differences in air flow between test tubes are hopefully cancelled out when the tubes are rotated, although this cannot be guaranteed. After making these changes, the percent variation between the controls was successfully reduced, in some experiments to as low as 5%.

During this project, the toxicity of fluconazole and terbinafine were tested singly on *P. subcapitata.* Terbinafine was highly toxic to the algae, with an EC_{50} based on growth rate of 90 nM, and based on biomass of 50 nM. The fact that there is a discrepancy in the values is normal. According to the OECD guidelines, the EC_{50} based on growth rate is generally higher than the EC_{50} based on biomass. The difference between the two values is not a measure of the sensitivity of this experiment, rather just that these two values are different. The use of the average specific growth rate for the calculation of the EC_{50} is preferred because it is dependent upon fewer variables. [31]

There is agreement between the data from the single-species assay and the SWIFT assay. In the SWIFT assay, the green algal species were more sensitive to terbinafine than the diatoms, as evidenced by the reduction in lutein content. In fact, the green algal species in SWIFT were slightly more sensitive to terbinafine than *P. subcapitata*. Based on the lutein content, terbinafine had an EC_{50} of 20 nM to the green algae. In contrast, the diatoms, as evidenced by the fucoxanthin content, were not affected in any of the treatments. There may have even been a slight stimulation of the diatoms at low concentrations. It would be interesting to conduct a single-species assay with a diatom to confirm this, and to determine if there is a range of concentrations at which diatom growth is inhibited by terbinafine.

The first experiments with fluconazole gave different results. When it was tested in the fall of 2009, toxicity was observed at 32 μM. While there was a very large discrepancy between the percent inhibition based on

biomass and growth rate at this concentration, inhibition was observed. Laboratory notes indicate that the 32 µM test tube was visually clearer, and less green, than the control test tubes. It is possible that nano-particles were present in the test tubes, and the combination of the fluconazole and the nano-particles inhibited the algal growth.

When the test was first repeated in the spring of 2010, no inhibition was observed, at concentrations ranging from 0.5 to 50 µM. Due to a lack of another explanation, new stock solutions were made, although it is unlikely that the fluconazole had degraded in the freezer in methanol over the course of approximately 5 months. The fluconazole experiment was repeated, first to a maximum concentration of 50 µM and then to 100 µM. The inhibition observed in both experiments was minimal, considering the high concentrations to which the algae were exposed. A graph showing the inhibition of average growth rate for both experiments is shown in Figure 45. For the March 2010 experiment, a statistically significant decrease in growth rate was observed at concentrations above 10 µM. For the April 2010 experiment, a statistically significant decrease in growth rate was observed at concentrations above 64 µM. At the highest concentration tested, 100 µM, the percent inhibition of growth rate was approximately 12%.

Levels of chlorophyll *a* were differently affected across the range of tested fluconazole concentrations. At intermediate exposure concentrations, levels of chlorophyll *a* were reduced, followed by an increase to higher exposure concentrations. A similar response in the violaxanthin, antheraxanthin and zeaxanthin content was also observed. This may be an artifact of the test methodology, for example the pigment extraction method, since the same trend was observed for several pigments. However, it is interesting to note that such bi-phasic responses of algae to exposure of azole-type fungicides have been reported previously.[18,37] The exact mechanisms are however unclear, but could be related to, for example, interference with pigment synthesis or growth-regulation systems.

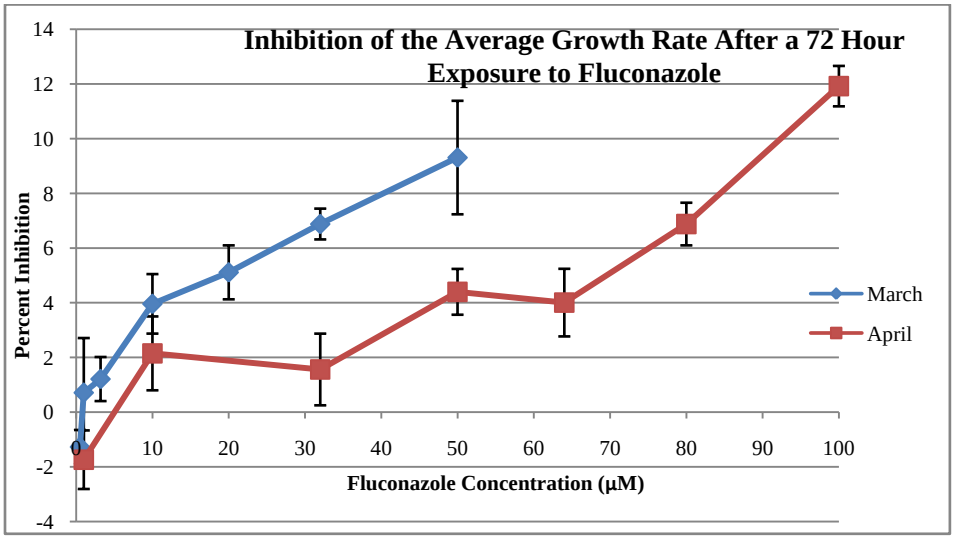

Figure 45. Graph comparing the results from the March and April 2010 *P. subcapitata* assay. In both experiments the algae were exposed to fluconazole. Each data point is an average of three replicates, with the exception of the April 2010 50 µM data point, which is an average of two replicates. The error bars represent one standard deviation.

The results from the fluconazole SWIFT experiment, though preliminary, do agree with the data from the single-species assay. The green algae did appear to be insensitive to the fluconazole treatments, especially when compared with the results from the terbinafine SWIFT assay. In addition, the diatoms did appear to be insensitive as well. It is difficult however, to determine whether any trends were observed, with the lack of replicates, and the lack of sterol data. However, a possible bi-phasic response in pigment levels over increasing concentrations of fluconazole would correspond with the observations from the test with *P. subcapitata*.

The sterols that had been previously identified in *P. subcapitata* were also identified in the control samples from both the terbinafine and the fluconazole experiments. Δ^7-chondrillastenol was previously identified as the most dominant sterol, accounting for approximately 65% of the sterol composition.[8] In the control samples, it accounted for 30 and 46% of the sterol composition. Ergostenol and Δ^7-chondrillastenol were also identified; however, the percentage of these sterols was very low (<10%). In the control samples, other sterols were identified, namely Δ8,22-ergastadienol, Δ7,22-ergastadienol , and 24,5α-cholesta-5,7,22-trien-3β-ol, all of which constituted between 10 and 25% of the sterol composition of the control samples. The structures of the tentatively identified sterols are shown in Figure 46.

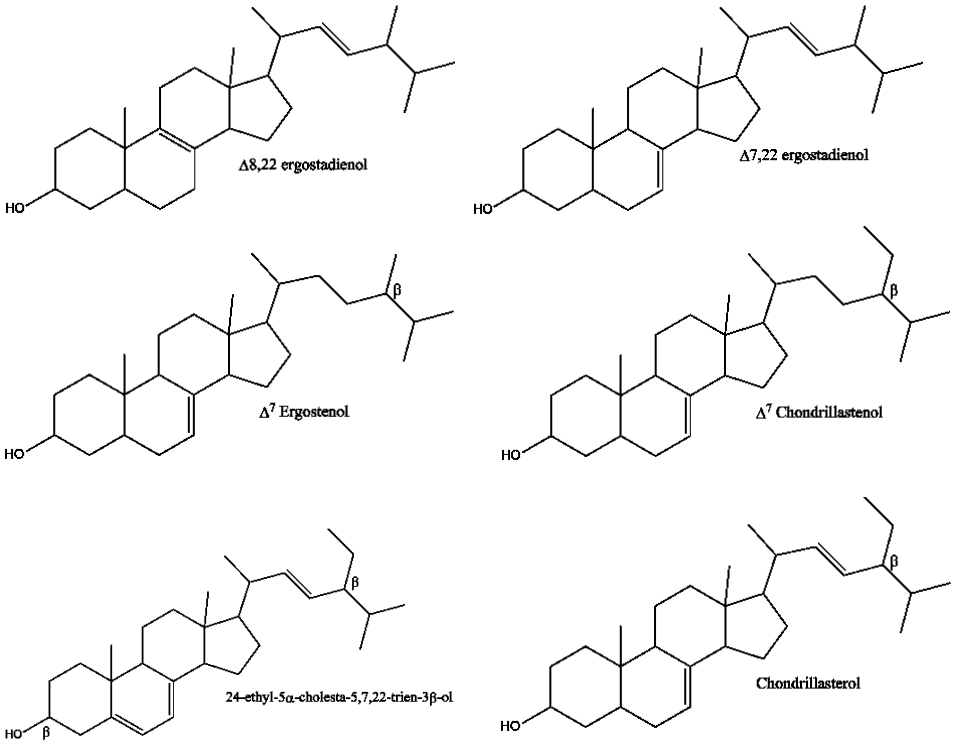

Figure 46. Sterols identified in *P. subcapitata*.

Terbinafine inhibits fungal growth by inhibiting squalene epoxidase, in the early stages of sterol biosynthesis.[35] In the presence of terbinafine, squalene is accumulated and other sterol intermediates are absent. Fungal cell death is believed to be due primarily to the accumulation of squalene, which may increase membrane permeability, and affect nutrient uptake.[35] To determine whether the mode of action is the same in algae, sterols were extracted at the end of the March 2010 terbinafine experiment and analyzed using GC/MS.

At low concentrations (at 20 and 32 nM), the presence of terbinafine stimulated the total sterol content, by over 40%. The reason for this occurrence is unclear; however, a stimulation at low concentrations in some phytosterols was also observed when marine algal communities were exposed to clotrimazole.[28] This is interesting because clotrimazole is an azole with the same mode of action as fluconazole,[28] but this response was seen with terbinafine and not with fluconazole (discussed below). However, it should be noted that changes in the sterol profile of a periphyton community can also result from changes in species composition, because algal species differ in their sterol composition. From 50 to 100 nM, a dose-response curve was observed, where increasing concentrations of terbinafine resulted in decreasing amounts of sterols. At 100 nM, the highest concentration tested, the total sterol content was reduced by over 40%. A dose-response curve was also determined for squalene. Squalene was first detected in the 10 nM sample. In the 100 nM treatment, squalene constituted over 80% of the sum of the squalene and sterol content. The decrease in normal sterols and the coinciding accumulation of squalene shows that inhibition of squalene expoxidase was the mechanism of action of terbinafine in *P. subcapitata*. Based on the coinciding effects on growth, it can be hypothesized that squalene accumulation was the mode of action for the algal cell death observed in the assay. No responses were detected in xanthophylls pigments ratios, which indicates that photosynthesis was not affected and that oxidative stress was not induced.

Fluconazole inhibits fungal growth by inhibiting cytochrome P-450-dependent 14α sterol demethylase (P-450_{DM}).[19] Fungi that have been exposed to fluconazole are depleted of ergosterol, and have an accumulation of 14α-methylated sterols, such as lanosterol.[19] The cyclization of squalene does not lead to lanosterol in algae, but rather to obtusifoliol.[24] If the mode of inhibition in algae is the same as in fungi, an accumulation of obtusifoliol and other 14α-methylated sterols would be expected. At 32 μM, the concentration of all six of the normal sterols was lower than half that of control level. For the majority of the sterols, the dose-response curve is flat above 50 μM. The only exception is chondrillasterol. The concentration of chondrillasterol actually increased above 64 μM; however, even at 100 μM, the concentration was still less than half of control level. As the concentration of the sterols decreased, with increasing fluconazole concentration, 14 α-methylated sterols were detected. Hence, this shows that the function of 14α-demethylase in *P. subcapitata* was inhibited in the presence of fluconazole. 14 α-methylated sterols were first detected in the 10 μM sample. Four 14 α-methylated sterols were detected, and the concentration of each increased with increasing fluconazole concentration. At the highest concentration tested, 100 μM, 14 α-methylated sterols represented 80% of the total sterol content. As would be expected based on the sterol biosynthesis pathway in algae, obtusifoliol was one of the 14α-methylated sterols detected, which increased in concentration as the fluconazole concentration increased. In addition, norlanosterol, which is a cholesterol precursor, was detected. This is interesting because cholesterol or cholesterol-like sterols (which do not have a side-chain at C-24) were not detected in the controls.

For terbinafine, the accumulation of squalene occurred simultaneously as the algal growth rate was inhibited. For fluconazole, growth inhibition, even at 100 μM, was minimal (<15%), and yet a considerable accumulation of 14 α-methylated sterols and a decrease in end-product sterols was observed. Hence, inhibition of 14α demethylase did not lead to detrimental effects on *P. subcapitata* growth.

Terbinafine was similarly toxic to its target species (fungi) and to *P. subcapitata* and Chlorophyceae algal species. Fungal inhibition is generally represented as the minimum inhibitory concentration (MIC), which is approximately the EC_{95} (the concentration required to inhibit 95% of growth). For terbinafine, MICs range from 9 nM to 300 μM for different fungal species.[34] In addition, the EC_{50} for ergosterol biosynthesis ranges from 1.5 nM to 212 nM for different fungal species.[34] For *P. subcapitata*, the EC_{50} for phytosterols was in the range of 64 to 80 nM. In contrast, fluconazole is more sensitive to its target species than to *P. subcapitata*

and freshwater algal species. MICs for fluconazole range from 0.13 μM to 326 μM for different fungal species.[38] While the highest concentration tested was only 100 μM, it is unlikely that even at 300 μM, an EC_{95} would be observed, especially for the Chlorophyceae communities.

According to the Swedish Chemical's Agency, the potential for a chemical to be hazardous in the environment is measured by acute and chronic tests.[11] This system is based largely on the system adopted by the European Union. Even though the 72-hour algal assay measures toxicity over several generations, it is considered an acute test. A substance is labeled in the acute category 1 if the EC_{50} to fish, crustacea, algae, or an aquatic plant is less than or equal to 1 mg/L. If a substance is part of acute category 1, it must be labeled as 'very toxic to aquatic life', it must be labeled with the word 'warning', and the pictogram, as shown in Figure 47, must be included. If acute toxicity is present and the substance exhibits a lack of rapid degradation or it is shown to bioaccumulate, then the substance is considered a chronic threat and is subject to a different set of warning labels.[11]

Figure 47. The placard that must accompany any substance categorized in acute category 1, according to the Swedish Chemical's Agency.[11]

An EC_{50} of 1 mg/L corresponds to an EC_{50} of 3μM for terbinafine. The EC_{50} for *P. subcapitata* based on growth rate was 90 nM, and the EC_{50} for the green algae in the SWIFT experiment was 20 nM. As such, terbinafine exhibits acute toxicity in the environment.

The placement of a chemical in Acute Category One does not depend on whether the chemical has been observed in the environment. In fact, little is known about whether terbinafine is present in the environment. Terbinafine concentrations have been measured at five WWTPs in Sweden, and while terbinafine was not identified in any of the WWTP effluents tested, it was identified in the sludge.[2] If the sewage sludge is land-applied, terbinafine may contaminate the environment. Toxicity data for terbinafine on terrestrial algal species (or any other algal species) was not found.

In addition, data on the presence of terbinafine in surface waters could not be found. It can be hypothesized that in locations where wastewater treatment is present, the concentrations of terbinafine in surface waters are minimal. However, there haven't been any studies to indicate whether terbinafine is retained in septic systems. In addition, if terbinafine were to be used in the future for veterinary use, a higher risk of contamination would be present.

In contrast, while fluconazole has been detected at very low nanomolar concentrations (<0.03 nM) in surface water,[3] and has been found to pass through WWTPs untreated,[2,3] it does not appear to be a risk to the freshwater algal communities tested or to *P. subcapitata*.

6.0 CONCLUSIONS

6.1 EXPERIMENTAL CONCLUSIONS

6.1.1 SINGLE SPECIES ASSAYS

- Terbinafine was very toxic to *P. subcapitata*, with an EC_{50} based on growth rate of 90 nM, and based on biomass of 50 nM. With a 95% confidence interval, a statistically significant decrease in growth rate was observed at concentrations above 20 nM.
- Fluconazole was much less toxic to *P. subcapitata*. An LOEC of 64 µM was observed; however, at 64 µM the percent inhibition of growth rate was less than 10%.
- Six sterols were identified in the *P. subcapitata* control samples, all of which are phytosterols: Δ8,22-ergastadienol, Δ7,22-ergastadienol, ergostenol, chondrillasterol, 24,5α-cholesta-5,7,22-trien-3β-ol, and Δ7-chondrillastenol.
- Terbinafine stimulated the total sterol content at low concentrations (20 and 32 nM). Between 50 and 100 nM, a dose-response curve was observed, as the total sterol content decreased with increasing concentration of terbinafine. Squalene was first observed in the 10 nM treatment. The percentage of squalene increased as terbinafine increased, to 85% of the total content, at 100 nM.
- Based on the results of both the assay and the sterol findings, terbinafine likely has the same mode of action in *P. subcapitata* as in fungi, where an increased presence of squalene leads to cell death.
- As the concentration of fluconazole increased, the concentration of all six end-product sterols decreased. At 32 µM, the concentration of all six of the sterols was below half of the control levels. Simultaneously, the concentration of four 14α-sterol intermediates increased. 14α-sterol intermediates were first observed in the 10 µM treatment. The fraction of 14α-sterol intermediates increased to a maximum of 0.8 in the 100 µM treatment.
- In the presence of fluconazole, a decrease in sterol content and an increase in 14α-sterol intermediates was seen at much lower concentrations compared to effects on growth. In fungi, the presence of 14α-sterol intermediates influences membrane permeability and function and inhibits cell growth. This does not appear to be the case with *P. subcapitata*.

6.1.2 SWIFT ASSAY

- The green algal species were very sensitive to terbinafine, but the diatoms were not. The green algal species had an EC_{50} based on lutein content, of 20 nM. The diatoms were stimulated at low concentrations of terbinafine (namely at the 10, 20 and 32 nM treatments), and were at or above control levels in the remainder of the treatments.
- The diatoms and green algal species were much less sensitive to fluconazole than to terbinafine. This observation is in agreement with the results from the *P. subcapitata* test.

6.2 OVERALL CONCLUSIONS

- Based on the results from the single-species assay and the SWIFT assay, terbinafine should be labeled as 'very toxic to aquatic life' due to its toxicity to *P. subcapitata* and Chlorophyceae algal communities.
- *P. subcapitata*, green algal communities, and diatom communities do not appear to be sensitive to fluconazole.

- As in fungi, the mode of action of terbinafine and fluconazole in *P. subcapitata* is inhibition of sterol biosynthesis at squalene epoxidase and 14α demethylase, respectively. It can be hypothesized that the inhibition caused by terbinafine resulted in cell death, due to increased levels of squalene. The inhibition of 14α demethylase did not inhibit the growth of *P. subcapitata*.

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APPENDIX ONE

Nutrient Stocks for Filtered River Water

Stock One:
9.34 grams $NaNO_3$
7.12 mL 0.5 M K_2HPO_4
Fill to 100-mL with MQ-H_2O

Stock Two:
4.057 mL 0.5 M $MgSO_4$
10 mL 0.5 M $Ca(NO_3)_2$
Fill to 100-mL with MQ-H_2O

Add to Filtered River Water
0.5 mL Stock One per liter filtered river water
0.5 mL Stock Two per liter filtered river water
40 µL 0.5 M $NaCO_3$ solution per liter filtered river water
1 mL Fe-EDTA solution per liter filtered river water
8 µL micronutrient solution per liter filtered river water