

**The effect of oxytetracycline on the response of *Lemna minor* growth to ultraviolet-B
radiation**

P.I.: Rachel Romero

Section C-04

T.A.: Scott Spal

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Abstract

Stratospheric ozone depletion due to anthropogenic pollutants such as chlorofluorocarbons (CFCs) has caused an increase in ultraviolet-B (UV-B) transmittance. Damaging UV-B effects are prevalent in surface aquatic plants such as *Lemna minor*. Surface aquatic plants are also affected by pharmaceuticals such as oxytetracycline (OTC). To determine the effect of OTC on the response of *L. minor* to UV-B radiation, a two-way factorial design used 0, 0.002, 0.02, and 0.2 mM OTC and ambient (5% ozone depletion) and elevated UV-B (15% ozone depletion). After two weeks, OTC significantly decreased mass change, frond number, and increased visible injury (chlorosis and necrosis). In contrast, the effect of UV-B was not significant. Furthermore, the effect of OTC did not depend on UV-B, possibly indicating that proteins and pigments affected by OTC are not related to those affected by UV-B. Future studies with greater replication, different UV-B levels, and dependent variables using physiological markers may be able to resolve the effect of OTC on the UV-B response. Independent of UV-B, the effect of OTC on *L. minor* impacts surrounding organisms that rely on the plant as a food source or require it for survival. If the effect of OTC on *L. minor* is representative of effects in other aquatic plants, aquatic ecosystems will be largely impacted.

Introduction

Stratospheric ozone has been diminishing since the 1970's due to anthropogenic pollutants such as chlorofluorocarbons (CFCs), chlorocarbons, and organobromides in the atmosphere (Björn 2007). Chlorofluorocarbons are released through industrial and commercial uses in aerosols, coolants, and electronic cleaning solvents. They remain stable in the atmosphere until they are broken down in the stratosphere by ultraviolet light, releasing chlorine as a by-product (Solomon 1999). Chlorine then reacts with ozone, causing depletion and a subsequent increase in ultraviolet-B (UV-B) radiation (Rowland 2006).

The increase of UV-B at the Earth's surface has direct effects on plants including DNA degradation, lipid breakdown, decreased photosynthetic rates, protein damage, and changes in morphology and growth (Young *et al.* 2006, Hader *et al.* 1998, Teramura and Sullivan 1994). In comparison to what is known about the effect of UV-B on terrestrial plants, relatively less is known about its effect on aquatic plants. Decreases in biomass and production of flavonoids (which usually function as a UV screen) have been observed in recent scientific studies (Rozema *et al.* 2002, Hader *et al.* 1998).

Due to both maximum surface UV radiation and shallow waters, free-floating plants in rivers and streams are potentially more vulnerable to UV-B damage (Williamson 1995, Hader *et al.* 1998, Germ *et al.* 2002). *Lemna minor*, commonly known as duckweed, is a free-floating plant found in temperate and subtropical regions around the world (Heide *et al.* 2006). Many animals require duckweed to survive or benefit from the plant blocking UV-B penetration lower in the water column (Scotland 1934). The plant has other functions including acting as a fertilizer supplement due to its high nitrogen content, indicator of water pollution due to its

sensitivity to heavy metals, and as a nutrient source (especially protein) for animals (Farooq *et al.* 2000, Islam *et al.* 2004).

Ultraviolet-B radiation has several negative effects on duckweed (Farooq *et al.* 2000, Farooq *et al.* 2005, Young *et al.* 2006). In two species of duckweed, *Spirodela polyrhiza* and *Lemna major*, high UV-B exposure caused chlorosis and necrosis, indicative of damage to the chloroplasts and the cell membrane (Farooq *et al.* 2000, Farooq *et al.* 2005). Ultraviolet-B radiation was also found to decrease biomass and change the concentrations of major fatty acids in *Lemna minor* (Young *et al.* 2006). Visible injury symptoms, another effect of UV-B radiation on *L. minor*, were a result of effects on chlorophyll, pigments, peroxidase, and carotenoids (Young *et al.* 2006).

In addition to UV-B exposure, aquatic ecosystems are also affected by pharmaceuticals in wastewater. Many pharmaceuticals have long half lives and are relatively long lasting. Furthermore, they are continuously added to the environment and may be harmful to surrounding ecosystems (Valverde *et al.* 2006, Ferreira *et al.* 2007). Oxytetracycline (5-hydroxytetracycline) is an antibiotic of the tetracycline family that is most frequently used in veterinary medicine for use against bacterial infections (Babic *et al.* 2006). Excretions from treated animals contain OTC and enter water sources through runoff (Babic *et al.* 2006). Other sources of OTC input into water include food pellets in fisheries which prevent antibacterial infections, and to a lesser extent human use (Ferreira *et al.* 2007, Valverde *et al.* 2006). Oxytetracycline has been found in United States streams at concentrations as high as 0.34 $\mu\text{g/L}$ (Ferreira *et al.* 2007).

The effect of OTC on plants is poorly understood. Tetracycline (which is similar in structure to OTC) causes chlorosis in some plants, possibly due to inhibition of translational activity of chloroplasts (Pro *et al.* 2003). Although the uptake of OTC by plants is not well

known, it is hypothesized to be energy dependent (Kong *et al.* 2007). Growth inhibition has been observed in terrestrial and aquatic plants including alfalfa (Kong *et al.* 2007), prasinophyte (Ferreira *et al.* 2007), and *Lemna minor* (Pro *et al.* 2003).

Oxytetracycline may affect the response of *L. minor* to UV-B radiation. Most plants ameliorate harmful UV-B effects through the accumulation of UV-absorbing compounds, such as anthocyanins, flavonoids, and hydroxycinnamic acids (Krause *et al.* 2007). Because OTC can inhibit protein synthesis, including chloroplast synthase, the effects of UV-B radiation may be amplified since the plant may not be able to protect itself (Pro *et al.* 2003). Furthermore, chloroplasts and protein are targets for UV-B damage as well, and may add to the damage done by OTC (Young *et al.* 2006). *Lemna minor* may be particularly affected by the two factors since it is a free-floating plant and exposed to pharmaceuticals from wastewater (Tripathi and Upadhyay 2003, Scotland 1934). Although recent studies have examined the harmful effects of OTC and UV-B, no studies have assessed the possible interaction of these factors. Therefore, the main objective of this study was to determine if the response of *L. minor* to UV-B radiation is affected by OTC. We hypothesized that under increased OTC and UV-B, there would be greater damage and growth inhibition.

Methods

To determine the effect of OTC on the response of *Lemna minor* to UV-B radiation, a two-way full factorial design was generated (Table 1). The first factor was OTC and had four levels: 0, 0.002, 0.02 and 0.2 mM. Levels were selected in order to simulate environmental levels and to include concentrations that cause measurable toxicity (Kong *et al.* 2007). The second factor, UV-B radiation, had two levels: ambient (grown in a greenhouse with current 5%

ozone depletion) and elevated (grown in a greenhouse with UV-B bulbs simulating 15% ozone depletion with 0.6534 kJ/m²/hr biologically effective UV-B). The elevated UV-B level was chosen since it is 5% greater than the predicted percentage for the year 2010 (Björn 1985). Each cell in the experiment contained 3 replications (n=3).

Each replicate began with 20 *Lemna minor* fronds which were rinsed with distilled water and weighed for an initial mass. Plants were placed in magenta boxes containing 250 ml of Hoagland's solution (Banerjee *et al.* 2008). Oxytetracycline was added as oxytetracycline hydrochloride to the Hoagland's solution in each magenta box, respectively (Sigma-Aldrich Company, St.Louis, MO).

Air pressure, relative humidity, and temperature were maintained with minimal differences for each UV-B level. Plants treated under ambient conditions were placed in a greenhouse with current 5% ozone depletion. Elevated UV-B treated plants were in a greenhouse with UV-B lamps and placed 38 cm above the ground to simulate 15% ozone depletion. The UV-B bulbs (with 0.6534 kJ/m²/hr biologically effective UV-B and an upper limit for irradiance integration of 320 nm) were wrapped with cellulose acetate to filter out wavelengths that do not reach the Earth's surface (Young *et al.* 2006). Bulbs were switched on for twelve hours daily, between 6:00 a.m. and 6:00 p.m. Magenta boxes were covered with cellulose acetate to help eliminate evaporation but still allow UV-B radiation to reach the plants.

Spring water was used to refill evaporated water since it is close to *L. minor*'s natural environment and can provide more nutrients than de-ionized water. The number of fronds in each replicate was counted to estimate growth and reproduction, since a frond is the plant itself and grows by asexual reproduction (Lemon *et al.* 2001). Another variable, visible injury symptoms of chlorosis and marginal necrosis, was observed because it has been caused by both

OTC and UV-B. Chlorosis was defined by any evidence of yellow appearance in the fronds. Marginal necrosis was defined by a brown discoloration at the edges of the fronds.

After two weeks (14 days), the final frond count and final visibly injured count was observed. Fronds were filtered from the magenta boxes and blotted with absorbent paper. A final fresh mass was obtained for each cell of the experiment. Data were analyzed using a two-way analysis of variance (ANOVA) through the software SAS JMP Version 5.1 (Statistical Analysis Systems 2007). Growth and visible injury were determined significant by a p-value < 0.05.

Results

Oxytetracycline significantly reduced fresh mass change ($F = 19.0685$, $p < .0001$). Lower concentrated replicates gained more mass than higher concentrated replicates (Figure 1). Independent of UV-B levels, the mean change in mass for 0 mM OTC treated plants was 3.66%, 340.60%, and 40500.00% greater than the 0.002, 0.02, and 0.2 mM plants, respectively (Figure 1). Mass change did not change significantly under UV-B and the effect of OTC did not depend on the level of UV-B radiation.

Frond number decreased significantly under higher OTC concentrations ($F = 32.2518$, $p < 0.0001$). Replicates treated with 0.02 and 0.2 mM OTC reproduced 57.04% and 506.54% less fronds than 0 mM replicates, respectively (Figure 2). However, the smallest concentration of OTC (0.002 mM) reproduced 3.91% more fronds than 0 mM replicates (Figure 2). In contrast to the effect of OTC on growth, the mean number of fronds reproduced did not change significantly under UV-B and the effect of OTC did not depend on UV-B levels.

Oxytetracycline significantly increased the percentage of visibly injured fronds ($F = 10.1556$, $p = 0.0005$). Plants treated with 0.2 and 0.02 mM OTC had 124.01% and 78.21% more visible injury than 0 mM replicates, respectively (Figure 3). In contrast, 0.002 mM plants had 27.23% less visible injury than 0 mM plants (Figure 3). The effect of UV-B levels on visible injury was not statistically significant and the effect of OTC did not depend on UV-B levels.

Other observations were made at the end of the two week experiment; however, we were unprepared to statistically analyze them. Replicates treated with lower concentrations of OTC produced much larger fronds than higher concentrated plants. The roots of 0.2 and 0.02 mM fronds turned black and many of them broke off easily. Oxytetracycline seemed to affect buoyancy of the plants as well. Fronds treated with 0 and 0.002 mM were able to recover more quickly than higher concentrated plants when pushed below the surface of the water with a toothpick.

Discussion

Our hypothesis, stating that under increased OTC, there would be greater damage and growth inhibition due to UV-B radiation, was not supported. This could indicate that the proteins or pigments affected by OTC are not related to those affected by UV-B.

Oxytetracycline, independent of UV-B, decreased growth and increased visibly injury. These results are likely due to the disruption of protein synthesis and affected chloroplasts. Replicates treated with 0.002 mM of OTC reproduced slightly more fronds and had less visibly injured fronds than 0 mM replicates. This suggests that 0.002 mM of OTC may not be toxic to *L. minor*. Because OTC contains nitrogen, it is also possible that a small amount is beneficial to the plant. Oxytetracycline seemed to affect the size of the fronds as well. Although we were not

prepared to obtain data on this variable, we noticed that replicates treated with lower concentrations of OTC produced larger and more buoyant fronds. Since stomata are on the upper surfaces of the plant, buoyancy is essential for survival (Scotland 1934).

Our results involving the effect of OTC were similar to previous studies which indicated growth inhibition in *L. minor*, alfalfa and prasinophyte (Kong *et al.* 2007, Ferreira *et al.* 2007, Pro *et al.* 2003). Prasinophyte, a microalgae, showed a significant reduction in growth when treated with OTC concentrations equal to or greater than 5.3 mg/L (Ferreira *et al.* 2007). Similar to our results, OTC inhibited alfalfa growth only at concentrations higher than 0.02 mM (Kong *et al.* 2007). Additionally, chlorosis was observed with increasing OTC levels (Kong *et al.* 2007). The brownish color, possibly due to OTC degradation, we observed in solutions after one day was also observed in Ferreira's study. Although studies have found that 90% of the antibiotic is recovered after 24 hours, OTC is photodegradable under daylight irradiation (Ferreira *et al.* 2007). Some OTC degradation products (reversible epimers) have been identified in other studies, but which metabolites are responsible for the color change is still unknown (Brain *et al.* 2005). The effect of these products on plants has not been determined, but they may have a differing effect than OTC.

Ultraviolet-B radiation did not have a significant effect on growth and visible injury. This contrast from other studies may have been caused by a slight difference in growth conditions such as temperature, pH, air pressure and humidity. A study on another species of duckweed, *Spirodela polyrhiza*, UV-B exposure produced visible injury symptoms (chlorosis and necrosis) and inhibited plant growth (Farooq *et al.* 2005). However, their UV-B levels consisting of no UV-B and ambient, low-dose UV-B (0.4 mW/cm²), differed from our levels, ambient and elevated UV-B (Farooq *et al.* 2005). Further studies may want to look further into

how seasonality affects growth inhibition. In another study, UV-B radiation did not affect *L. minor* growth during the summer, but significantly inhibited growth during the fall (Young *et al.* 2006). It was suggested that the summer duckweed was exposed to higher UV-B radiation and was more resistant to UV-B.

Future studies may want to observe physiological markers such as frond size and chlorophyll content. Products of OTC degradation responsible for color changes in water should be identified as well as their biological effects. Although our data was not statistically significant, we noticed that replicates treated with lower OTC concentrations had less visibly injured fronds and within each OTC concentration, the elevated UV-B treated replicates had more visibly injured fronds. This suggests that a possible interaction could have a greater effect on visible injury symptoms such as chlorosis and necrosis than on growth. Further studies with greater replication may be able to resolve the effect of OTC on the response to UV-B radiation.

Our results concur with other studies on the effect of OTC on plants, adding to this less known field of study. A variety of ecological consequences follow from the detrimental effects observed in *L. minor*. A decrease in duckweed growth affects many animals that require the plant for survival either as a food source or as a habitat for developmental stages (Scotland 1934). In addition, organisms that are lower in the water column may be exposed to more UV-B radiation in the absence of a floating mat of duckweed at the surface. If our results in *L. minor* are representative of effects in other aquatic plants, then many aquatic ecosystems will be greatly affected. Aquatic plants in ecosystems closest to industrial and hospital effluents, sewage treatment facilities, and aquacultures are exposed to highest amounts of pharmaceuticals including OTC (Ferreira *et al.* 2007). Aquatic organisms may lose their food source, residency, or source of protection from UV-B radiation. Some terrestrial plants may even be affected by

OTC. In areas where water runoff is prevalent, OTC has been found in liquid manure at concentrations as high as 4 mg/kg and 198.7 µg/kg in top soil (Brain *et al.* 2005). Similar to aquatic ecosystems, growth inhibition in these plants is likely to affect surrounding organisms.

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Table 1. The two-way full factorial design with two levels of UV-B radiation and four levels of OTC concentration.

	Oxytetracycline concentration (mM)			
UV-B radiation	0	0.002	0.02	0.2
Ambient UV-B (regular greenhouse)	n=3	n=3	n=3	n=3
Elevated UV-B (15 % ozone depletion, 0.6534 kJ/m ² /hr)	n=3	n=3	n=3	n=3

Figure Legend

Figure 1. Mean change in *Lemna minor* fresh mass (+/- standard errors) in the 8 cells of the two-way factorial experiment with factors of UV-B, ambient (5% ozone depletion) and elevated (15% ozone depletion), and OTC concentration, 0 mM, 0.002 mM, 0.02 mM, and 0.2 mM.

Figure 2. Mean number of *Lemna minor* fronds reproduced (+/- standard errors) in the 8 cells of the two-way factorial experiment with factors of UV-B, ambient (5% ozone depletion) and elevated (15% ozone depletion), and OTC concentration, 0 mM, 0.002 mM, 0.02 mM, and 0.2 mM.

Figure 3. Mean percentage of visibly injured *Lemna minor* fronds (+/- standard errors) in the 8 cells of the two-way factorial experiment with factors of UV-B, ambient (5% ozone depletion) and elevated (15% ozone depletion), and OTC concentration, 0 mM, 0.002 mM, 0.02 mM, and 0.2 mM.

Figure 1

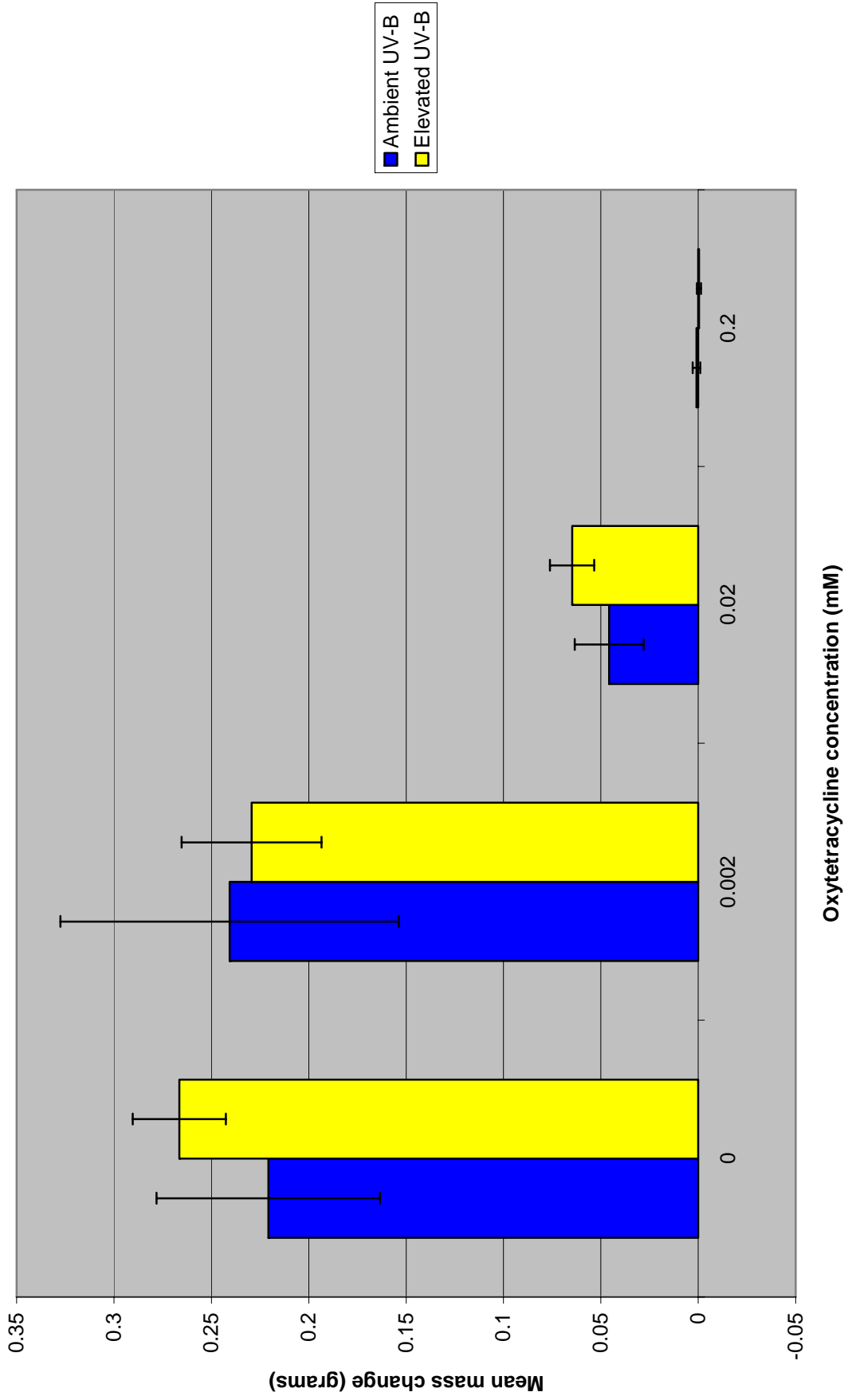


Figure 2

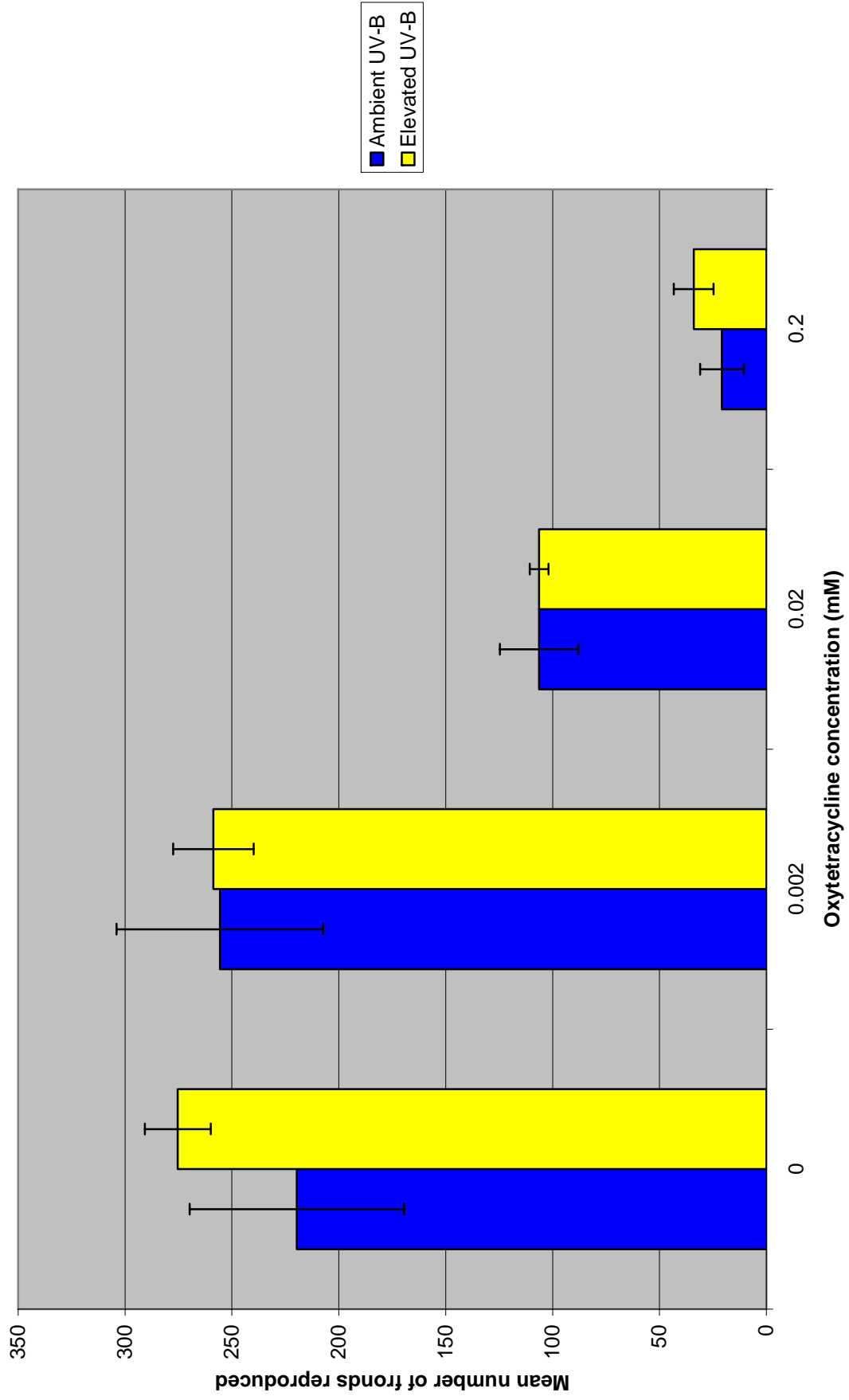


Figure 3

