INVESTIGATING THE SUB-ACUTE RESPONSES OF *LEMNA MINOR*, *PSEUDOKIRCHNERIELLA SUBCAPITATA*, *EUGLENA GRACILIS* AND *ANODONTA GRANDIS* TO TRIBUTYLTIN-HYDRIDE AND ATRAZINE IN THE DEVELOPMENT OF AN EARLY-WARNING BIOMONITORING SYSTEM TO RAPIDLY DETECT SOURCE-WATER CONTAMINANTS

by

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Abstract

Investigating the Sub-acute Responses of *Lemna minor*, *Pseudokirchneriella subcapitata*, *Euglena gracilis* and *Anodonta grandis* to Tributyltin-hydride and Atrazine in the Development of an Early-warning Biomonitoring System to Rapidly Detect Source-water Contaminants

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Freshwater resources exist in limited quantities and are subject to increasing demands due to the consumption by residential, commercial and industrial uses. There are concerns that the widely used chemical analysis of drinking water does not deliver timely results. This study examines the efficacy of developing a holistic, multi-organism early-warning biomonitoring technology to assess aquatic toxicity. Sensitive indicator species such as *Lemna minor, Pseudokirchneriella subcapitata, Euglena gracilis* and *Anodonta grandis* have been selected due to their specific behavioural and short-term biochemical responses in the identification of classes of contaminants in aquatic environments. Tributyltin, an antifouling agent in paints used on boats and atrazine, an herbicide widely used on agricultural crops, are evaluated in increasing concentrations to identify behavioural changes in these organisms. These graded responses, upon implementation in models, will warn water treatment operators of incoming contaminants and help identify the nature of the stressor. All organisms displayed some sensitivity to selected concentrations of the two test chemicals. The normal growth rate of *L. minor* dramatically declined with exposure to TBT (100.0 µg/L) and atrazine (500.0 µg/L). Monitoring the biochemical changes, dissolved oxygen production, and also the growth rate, cell counts, of *P. subcapitata* showed significant effects to similar concentrations of TBT (100.0 µg/L) and atrazine (500.0 µg/L). The aquatic protist, *E. gracilis*, alters its cell morphology in the presence of low concentrations of TBT (10.0 µg/L) and atrazine (50.0 µg/L). Respiration patters of the bivalve, *A. grandis*, was directly influenced by the two chemicals, TBT (1.0 µg/L) and atrazine (50.0 µg/L). This study demonstrates that biological assessments of water samples deliver a rapid, realistic representation of the surrounding aquatic environment conditions.

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List of Abbreviations

ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
ASTM	American Society for Testing and Materials
ATZ	Atrazine
AWWA	American Waterworks Association
BCF	Bioconcentration Factor
BEWS	Biological Early Warning System
$CaCl_2$	Calcium dichloride
CCD	Charged-Coupled Device
DF	Delayed fluorescence
DMSO	Dimethyl-sulfoxide
DO	Dissolved oxygen
EC_{50}	Effective concentration of a chemical effecting 50% of the test population
EDCs	Endocrine disruptor
IMO	International Maritime Organization
LOEC	Lowest observable effect concentration
MCLs	Maximum concentration levels
MFB	Multispecies Freshwater Biomonitor
NOEC	No observable effect concentration
OD	Optical Density
OECD	Organization for Economic Cooperation and Development
PAH	Polynuclear aromatic hydrocarbons
PPCPs	Pharmaceuticals and personal care products
PSI	Photosystem I
PSII	Photosystem II
SD	Standard deviation
TBT	Tributyltin
USEPA	United States Environmental Protection Agency

UV/VIS	Ultraviolet/Visible light
WTP	Water Treatment Plant
WWTP	Wastewater Treatment Plant

CHAPTER 1

INTRODUCTION

Global freshwater supplies are either surface or ground waters that are found in the natural environment and exist in limited quantities (Sabik *et al.*, 2000; Anderson, 2003). Protection of this natural resource is of great concern due to uneven global distribution and most importantly the possibility of contamination from point and non-point sources (Dorigo *et al.*, 2004). In developed countries, industrial sources result in increased water consumption and an increased production of wastes. The improper disposal and treatment of these wastes can result in contaminated drinking water sources and ultimately pose human health risks (Metcalfe *et al.*, 2003). Another problematic scenario, initiated by the Western world, is the unnecessary large consumption of clean freshwater. Attempts to enforce water conservation practices, through innovative technologies and awareness, have been ongoing to reduce the excessive use of this resource (Anderson, 2003; Sala, and Serra, 2004). Water usage originates from a variety of sources such as industrial and commercial practices, domestic uses, public facilities and general leakage (Gleick, 2000). Identifying and creating awareness of this demand draws awareness in government agencies, policy makers and stakeholders to ensure safe drinking supplies in their jurisdictions (Gleick, 2000). Limited water treatment technologies in developing countries introduce further concern for contaminated waters (Loucks, 2000). Unsafe drinking water in developing countries affects a large population and results in poor health conditions in the average individual living in these geographic locations. Development of a cost-effective technology to rapidly identify and treat contaminants in drinking water sources is an ideal answer to reduce health risks, ensuring potability, and would dramatically improve quality of life in both developed and developing countries. Such technologies include but are not limited to ion exchange, reverse osmosis, granular activated carbon and air stripping (Goodrich and Lykins 1991).

Chemical analyses are the most common methods used to identify components found in water samples (Ministry of the Environment, Ontario, 2008a). In Ontario, required chemical test methods are outlined in the "Protocol of Accepted Drinking-Water Testing Methods" (Ministry of the Environment, Ontario, 2008a). Also, stated in this document, there are other chemical standard methods set out by other agencies such as the United States Environmental Protection Agency (USEPA), American Waterworks Association (AWWA), ASTM International (formally the American Society for Testing and Materials), and AOAC International (formally the Association of Official Analytical Chemists) (Ministry of the Environment, Ontario, 2008a). This practice has been shown to be very effective; however, a limiting factor is the extensive time required to deliver results. When a water sample is extracted from a source, it will undergo multiple chemical tests to determine if a contaminant is present or absent. If a particular contaminant is present, further investigation is conducted to determine if the concentration exceeds the regulatory standards set out by officials. Another limitation of this process is that it does not account for all contaminants. Only a selection of known toxicants have been identified and treated in the chemical approach which are outlined in Drinking Water Act, 2002 (Ministry of the Environment Drinking Water Ontario, 2008b). A number of contaminants are not accounted for, such as certain metals, pesticides, polynuclear aromatic hydrocarbons (PAHs), halogenated organic compounds, pharmaceuticals and personal care products (PPCPs), endocrine disruptors (EDCs) and also xenobiotics (Synder *et al.*, 2003; McCarthy *et al.*, 2007). Another area of potential concern is contamination of drinking water sources with biosolids via runoff from agricultural lands. Biosolids are processed nutrient rich byproducts derived from domestic and commercial sewage (Topp *et al.*, 2008). This treated sludge is produced at wastewater treatment plants (WWTP) and have been deemed safe for application on agricultural crops as an alternative natural nutrient rich fertilizer (Topp *et al.*, 2008). The treatment process of this resource removes regulated components but poses major concerns due to the release of unregulated concentrations of PPCPs and other chemicals into the aquatic environments via runoff (Topp *et al.*, 2008). The application of sludge can be either surface or injected into the soil. The surface application is susceptible to

increased runoff rates while the injection reduces these rates dramatically (Topp *et al.*, 2008).

In contrast to the well-known chemical tests is a biological approach that can deliver comparable results. The use of biological monitoring of living organisms through monitoring of behavioural characteristics has been applied in the past (Giesy and Allred, 1985). A historical example of biological warning systems is the monitoring of a canary's behaviour during extraction of raw material in deep caves. This sensitive indicator species would deliver an early-warning signal to miners indicating unsafe air quality in the enclosed mines. The use of bioassays has shown to be a reliable tool to rapidly identify contaminants present in the surrounding environment (Giesy and Hoke, 1989). The use of an early-warning biomonitoring is not a new concept and a number of technologies have been developed in Europe (Gerhardt *et al.*, 2002, Tahedl and Häder, 1999, and Streb *et al.*, 2002). Germany is a leader in developing these systems to identify unsafe aquatic environments for a number of years. Early-warning biomonitoring systems have not been heavily studieded in North America, where water resources are significantly high. Traditional chemical testing may not deliver sufficient timely analysis to indicate safe water conditions. Biological assessments deliver the advantage of identifying hazardous concentrations of chemicals at the sub-acute level, less than 24 hours, by expressing rapid behavioural responses. This allows for the identification and treatment of water-borne toxic substances before end users would be exposed.

In this study, a selection of suitable organisms which delivered the best responses to environmentally-relative concentrations of contaminants found in freshwater sources was investigated. Laboratory tests involved dose-dependent experiments with two chemical contaminants, tributyltin-hydride (TBT) and atrazine. Changes in behaviours such as cell shape and normal movement and other endpoints such as changes in dissolved oxygen and cell growth were monitored to compare with normal parameters for allocated time periods. This research will lead to the development of a free-flow *in situ* model that will utilize multiple indicator organisms to automatically detect unfavourable aquatic conditions through automated monitoring of organisms' behaviours. If the system

detects contaminated samples and identifies the culprit class of contaminants, an engineering solution will be set in place to identify and extract harmful contaminants from the water source.

The objectives of this thesis are as follows:

- to select a battery of sensitive indicator organisms,

- to design experimental procedures monitoring sub-acute responses to chemical stressors and determine quantifiable stress responses, and

- to determine the most sensitive endpoints for individual organisms to further develop an early-warning biomonitoring system.

The overall objective for the project is:

- to compliment current chemical analysis with a holistic biological approach by incorporating the rapid responses of these sensitive indicator organisms to aquatic contaminants in detecting unsuitable freshwater samples.

All thesis objectives stated above were met and are presented in the following document. This research will aid to attain the overall project objective to develop an aquatic early-warning biomonitoring system.

CHAPTER 2

LITERATURE BACKGROUND

2.1 Ontario Drinking Water Standards

Drinking-water standards in Canada are set out by the individual provincial governments. Ontario Drinking Water Quality Standards (O. Reg 169/03) outlines the allowable limits of 158 chemical/physical, microbial and radiological parameters (Appendix A). These standards were developed from the Safe Drinking Water Act of 2002. The main reason for O. Reg. 169/03 is to standardize the allowable levels of substances which have been proven to pose health risks to end users. Safe drinking water for human consumption must have little or none of the following; disease-causing organisms, toxic chemicals, or radioactive materials. Other characteristics of drinking-water such as odour, taste, turbidity and colour are controlled to make it more pleasant to consume. The Ontario standards does regulate the allowable concentrations of atrazine but does not have set standards for Tribytyltin (TBT) limits. In the guidelines, atrazine had allowable concentration of 0.005 mg/L (Safe Drinking Water Act, 2002). It is difficult to detect and regulate unwanted substances found in water sources but it is important to take a proactive approach in reducing contamination rather than taking action following a disaster. Many incidents concerning water contamination have taken place even in developed countries. One example is in Walkerton, Ontario, where the drinking water became contaminated from *Escherichia coli* and *Campylobacter jejuni* bacteria without detection where thousands became ill and seven people died in 2000 (Prudham, 2004; Richards, 2005). Current increases of waste production facilitate the importance of water treatment and regulation of all potential contaminants present in drinking water sources.

2.2 Indicator Species

Giesy and Allred (1985) have conducted bioassays using a battery of organisms to determine environmental toxicity conditions. Their research using multispecies showed to be an ideal approach to identify unfit environmental conditions. By monitoring the parameters of more than one species allows the observer to identify toxic impacts of a more representative population found in the environment (Giesy and Allred, 1985). The main goal of using a multispecies model is to determine the effects at the ecosystem-level which is more representative of the natural environment (Giesy and Allred, 1985). Isolating individual indicator species does not incorporate tropic interactions amongst organisms which occur regularly with overlapping populations.

A number of aquatic plants and a single animal species were included in the biological-based approach of this project for a number of reasons. Firstly, they all play a key role in aquatic environments, especially with regards to their involvement in nutrient cycling. They have proven to be ideal indicator species displaying short-term response characteristics in the presence of contaminants. The organisms selected were emergent macrophyte *Lemna minor,* the green algae *Pseudokirchneriella subcapitata,* the single celled protist *Euglena gracilis,* and the freshwater bivalve *Anodonta grandis*. Each organism underwent toxicity experiments in the laboratory to monitor their sensitivity to the selected chemicals of concern, TBT and atrazine. Further experimentation using other chemical toxicants should be conducted and selected from a list of known compounds tested in drinking water by the Ontario government based on their ability to persist in the environment (Appendix A). These toxicity experiments are necessary to determine the organisms' sensitivity to a particular chemical and determine if they show stress responses within acceptable limit.

2.2.1 *Lemna minor*

Lemna minor, common name "duckweed", is from the family Lemnaceae (Wang, 1990). It is a free floating surface macrophyte found in many freshwater environments. *L. minor* has a doubling time of approximately 1.4 days and has been recommended as a standard test species in freshwater environmental studies (Wang, 1990). It is composed of

two segments, 2-3 fronds, which are approximately 2 mm in length, and a single root, ranging 5-20 mm, (Figure 2.1) which is submerged in the water column (Wang, 1990). *L. minor* is capable of living in a pH distribution of 5-9 and optimally lives in waters with pH of 6.5-7.5 (Wang, 1990). These vascular angiosperms can be found in temperatures ranging from 6 to 33^{o}C (Wang, 1990). Reproductive behaviour is vegetative, minimizing genetic variability and producing identical offspring (Hillman, 1961; Moody and Miller, 2005). *L. minor*'s life cycle involves rapidly dividing colonies and if temperatures fall below 6-7^{o}C, parent plants will produce a starch-filled capsule, termed a turion, for winter survival (Wang, 1990). These capsules will sink to the sediments and when the temperatures become favourable they will open and return to the surface. *L. minor* is considered an important nutrient source of protein and fat for some birds and fish species (Hillman, 1961).

Moody and Miller (2005) identify that *Lemna* species have been used in toxicity experiments since the early 1930s and has led to the development of current environmental guidelines and legislation. Many governmental authorities have adopted plant toxicity tests for environmental assessments and monitoring (Wang and Freemark, 1995). Tests using duckweed as an indicator species have been recommended under The Federal Insecticide, Fungicide and Rodenticide Act (1982) and The Toxic Substance Control Act (1985) in the United States (Wang and Freemark, 1995). Also, duckweed is a recommended test organism in Canada for pulp and paper effluent regulations (1992) under The Fisheries Act (1941) (Wang and Freemark, 1995). The Organization for Economic Cooperation and Development (OECD) has set out "The Guidelines for the Testing of Chemicals: *Lemna* sp. Growth Inhibition Test", which is a seven day growth test protocol assessing the number of fronds and also monitoring plant biomass (total frond area, dry weight or fresh weight) (OECD, 2002a). These tests compare samples with a control to determine effective concentration of 50% inhibition (EC_{50}), lowest observable effect concentration (LOEC) and no observable effect concentration (NOEC) (OECD, 2002a). Environment Canada has also published a set of standard test methods, "Biological Test Methods: Test for Measuring the Inhibition of Growth Using the

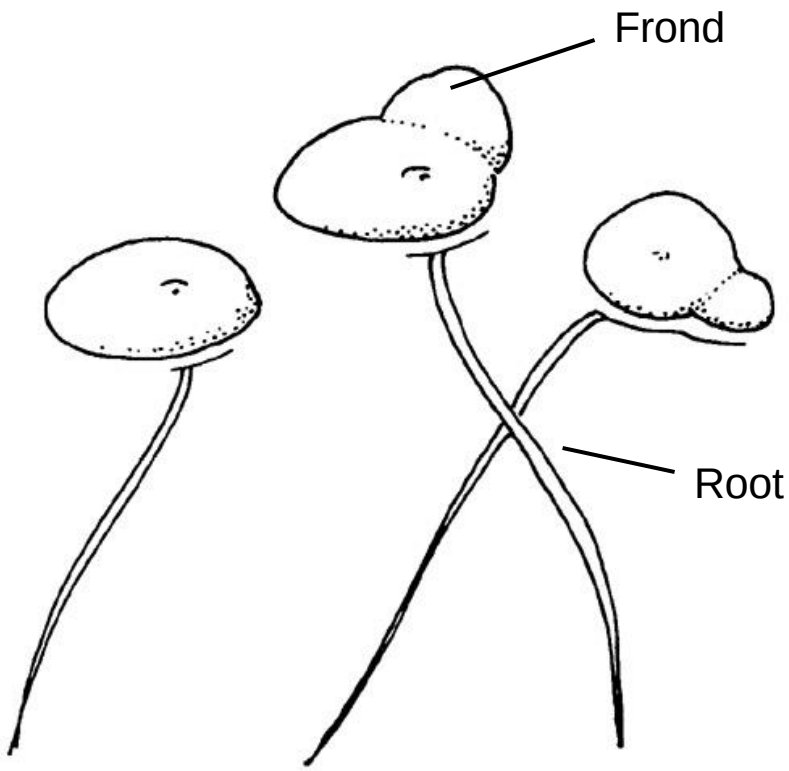


Figure 2.1: Line drawing of the emergent macrophyte *Lemna minor* (University of Florida, 2008).

Freshwater Macrophyte, *Lemna minor*" (Environment Canada, 2007a). The United States Environmental Protection Agency (USEPA) also has their own modifications for duckweed standard testing and is outlined in the "Ecological Effects Test Guidelines: Aquatic Plant Toxicity Test Using *Lemna* spp., Tiers I and II" (USEPA, 1996a). Standard tests from the above-mentioned agencies have slight modifications, such as different growth medium but in general the tests are quite similar. The duration of the experiments are all seven-day tests and the measured endpoints are all standard.

L. minor has been used in the laboratory as an environmental toxicity biological indicator due to its ease in culturing, low-cost maintenance, small size, rapid growth rate, and its multiple endpoints to determine the effects of aquatic contaminants (Hillman, 1961; Wang, 1990; Moody and Miller 2005). It has been used to detect heavy metal toxicity, organic compounds such as pentachlorophenol and also herbicide pollution (Wang, 1990). Previous experiments which have used *Lemna* include growth inhibition tests which include frond counts, measuring dry-weight biomass and root length measurements (Fenske *et al.,* 2006; Moody and Miller 2005). Previous toxicity experiments indicated that frond counts over time delivers the most accurate results in comparison to alternative tests (Hillman, 1961; Bishop and Perry, 1981). Dry-weight measurements mainly represent starch content and therefore produce inaccurate results with respect to growth rates (Hillman, 1961). Past experiments do not identify sub-acute stress responses and therefore this research looked at how selected chemicals affected *L. minor*'s growth on a sub-acute level. Another rapid endpoint which could be identified is monitoring the consumption of carbon dioxide and oxygen evolution. Chemical contaminants can have an effect on the light and dark reactions of photosynthesis impeding the normal growth of this plant species.

2.2.2 *Pseudokirchneriella subcapitata*

Pseudokirchneriella subcapitata (formally *Selenastrum capricornutum*), is non-motile, unicellular, crescent-shaped green algae (Figure 2.2) that can be found in many freshwater environments (Fairchild *et al.*, 1997). It is an important primary producer and

therefore is actively involved in the cycling of nutrients in aquatic ecosystems. This diploid organism grows quickly with a doubling time of 18-20 h which makes it an ideal indicator species for monitoring sub-acute parameters (Fairchild *et al.*, 1997). It has been heavily used in toxicity experiments due to its ease of culturing in the lab, its sensitivity to contaminants and low cost experiments (Katsumata *et al.*, 2006). Due to its high sensitivity and primary productivity, this organism is highly important to the success of the ecosystem in which it lives.

Tests with *Pseudokirchneriella subcapitata* have been in use since the 1970s for assessing aquatic eutrophication which led to the development of standardized tests used today (Fairchild *et al*., 1997). Algae toxicity test have been used globally and many standard test protocols have been developed by agencies to maintain uniformity when tests are conducted. The OECD has developed a document, "OECD Guidelines for the Testing of Chemicals: Freshwater Algae, Cyanobacteria, Growth Inhibition Test" which is a 72-h test monitoring the exponential growth of algae (OECD, 2002b). Environment Canada has also developed their version of standard methods for toxicity tests using algae, "Biological Test Methods: Growth Inhibition Tests Using a Freshwater Alga" (Environment Canada, 2007b). The USEPA collaborated their own document with standard toxicity tests which are outlined in "Ecological Effects Test Guidelines: Algal Toxicity, Tiers I and II" (USEPA, 1996b). All three agencies follow similar methods with minor alterations. All three methods investigate growth inhibition experiments and also the test period is either 72-h or 96-h.

Kaneko *et al.* (2004) used phosphorus uptake by green algae as a short term toxicity parameter. Phosphate is a limiting resource in freshwater environments and is an essential macronutrient used by plant species for growth and development. In toxin-free waters green algae will take-up a constant amount of available phosphorus for normal development and this process can be measured using phosphate chemical analysis (Kaneko *et al.*, 2004). Introducing different concentrations of a contaminant will have a direct effect on the phosphorus uptake by the plant. Increasing concentrations of zinc

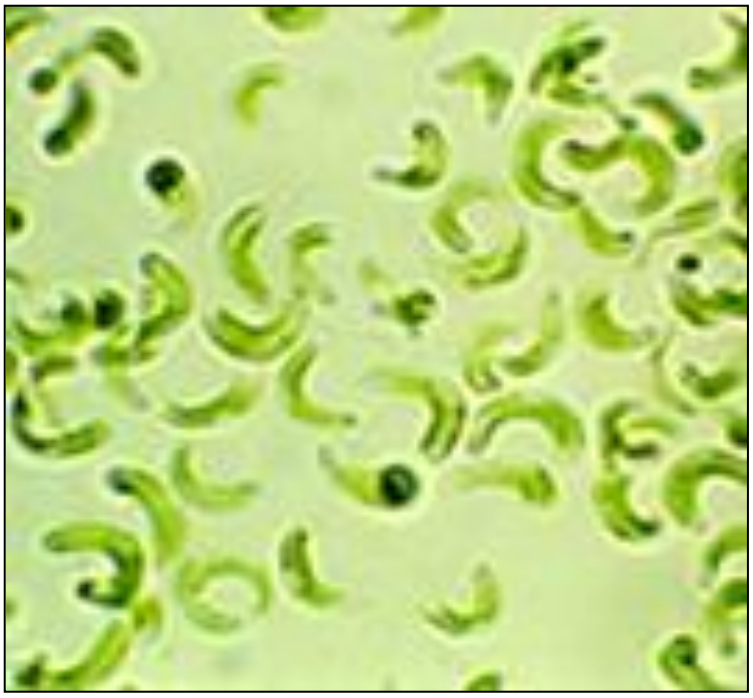


Figure 2.2: Photograph of the green algae *Pseudokirchneriella subcapitata* (Aquatic Habitat Management. 2008).

nitrate shows an increase in inhibition of phosphorus uptake by *P. subcapitata* (Kaneko *et al.*, 2004). The test period for this experiment was approximately 3-h but was indicated by the authors that this time frame could be significantly reduced by altering the cell inoculation period (Kaneko *et al.*, 2004). Another method to monitor phosphorus concentrations rapidly would be to incorporate a phosphorus probe to monitor fluctuations in real time.

Besides changes in biomass, the following biochemical changes can be used for monitoring rapid changes under stressed conditions. Delayed Fluorescence (DF) is a parameter which is used to determine photosynthetic output (Katsumata *et al.*, 2006). Many contaminants in the environment can affect the light reaction of photosynthesis and in particular the electron transport chain between photosystem II (PSII) and photosystem I (PSI) (Katsumata *et al.*, 2006). These two photosystems are reaction centers, made up proteins located in the chloroplasts, which absorb light energy to produce oxygen as a by-product (Allen, 2003). Photosystem II occurs first and is the driving mechanism for the electron transport chain while PSI provides energy used by the plant for growth and other functions (Wraight and Crofts, 1971). When light is absorbed on the antennal complex (reaction center) of PSII, electrons are transferred to a primary acceptor at the beginning of the electron transport chain (Allen, 2003). This reaction promotes energy flow towards the Calvin cycle facilitating the plant with energy and sugars for growth (Allen, 2003). DF occurs when a plant is suddenly placed in dark conditions and electrons will return to PSII to re-excite the reaction center, where fluorescence occurs for a set period and then decays over time (Strehler and Arnold, 1951). DF can be used to detect growth inhibition in green algae. For example, if *P. subcapitata* is exposed to certain concentrations of a contaminant, DF timings will be altered when compared to normal conditions. Katsumata *et al.* (2006) showed that results from a 15 minute exposure to contaminants such as simazine and 3,5-dichlorophenol, DF had similar results when compared to a 72-h growth inhibition test.

2.2.3 *Euglena gracilis*

Euglena gracilis (~15µm) is a single-celled protist, containing a flagellum (Figure 2.3) for motility (Gajdosova *et al.,* 1996). This species has unique plant and animal characteristics which provides many physiological endpoints such as phototaxis (orient due to light) and gravitaxis (orient due to gravity) (Ohta *et al.*, 1999; Streb *et al.,* 2002). Its flagellum facilitates motility for the organism within the water column and efficiently captures nutrients. The chloroplasts allow the protist to perform photosynthetic reactions.

Historically, *Euglena* species have been studied for their phototaxis capabilities. In the mid-1970s, Checcucce *et al*. (1976) mentioned the early work of Jennings (1906) who looked at the photoorientation mechanism of *Euglena*. Checcucce *et al.* (1976) investigated the ability for three different species of *Euglena* to orient towards light and concludes that the eyespot facilitates manoeuvrability. ECOTOX, developed by Tahedl and Häder (1998), is an early-warning biomonitoring system which can observe, analyse and produce accurate results from the behavioural changes of microscopic organisms. ECOTOX uses real-time image analysis to monitor the behaviours of *E. gracilis* using video cameras to determine stress levels compared to the organism's normal behaviour (Streb *et al.,* 2002). Its ability to photosynthesize could also be monitored as a short-term endpoint, observing changes in dissolved oxygen, similar to that of *Pseudokirchneriella subcapitata* (Ohta *et al.,* 1999). Under stressed conditions, this particular organism changes its shape from its normal elongated spindle conformation to a constricted cyst form and also loses its flagellum (Lonergan and Williamson, 1988; Ohta *et al.,* 1999). *Euglena* has been shown to be sensitive to certain concentrations of tributyltin-chloride (TBT-Cl) which reduces it motility (Ohta *et al.*, 1999). Microtubules found in the cytoskeleton of the organism either polymerise increasing the body asymmetry (elongated) or depolymerise resulting in the cyst shape (Lachney and Lonergan, 1985).

2.2.4 *Anodonta grandis*

Anodonta grandis (Figure 2.4), common name "giant floater", is a freshwater bi-valve filter feeder native to North America and is an important animal in the food web

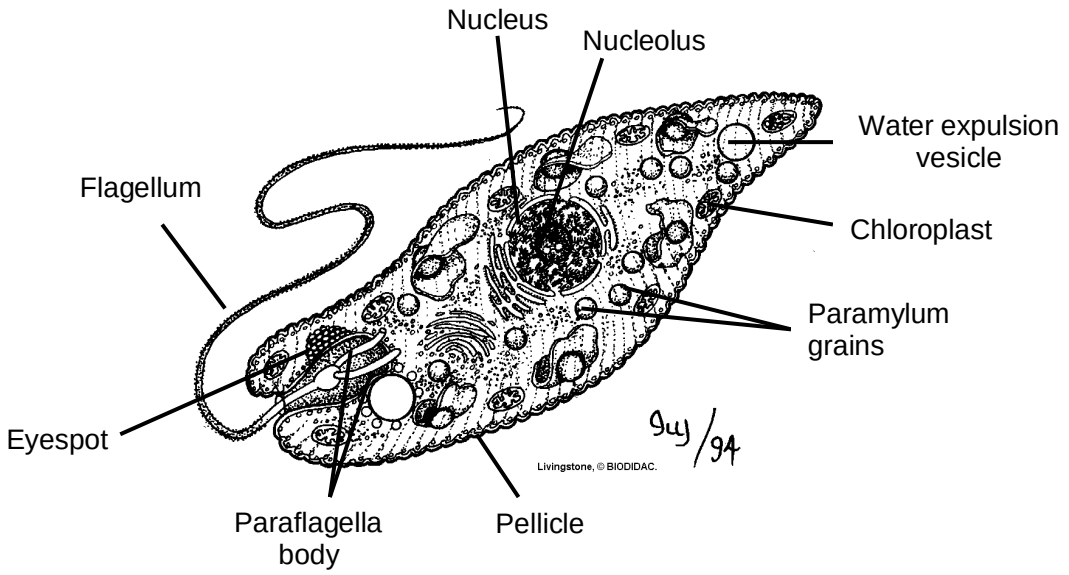


Figure 2.3: Line drawing of the single-celled protist *Euglena gracilis* (BIODIDAC. 2009a).

(Mackie, 1991). This organism is an herbivore filtering and consuming suspended particulate matter found in the surrounding waters (Mackie, 1991). It is a member of the benthic community which remains on, or slightly burrowed in, the sediments of both slow-moving lentic (lakes or ponds) and faster moving lotic (streams or rivers) freshwaters (Mackie, 1991). Primary predators include turtles, birds, some fish species and also terrestrial mammals such as raccoons. Its shell length, from posterior to anterior end, can be up to 15cm (Byrne and McMahon, 1991). Reproductive processes include the release of sperm from the male into the water column which enters the siphon of the female to fertilize eggs in a brood pouch located on the gills of the female mussel (Texas Parks and Wildlife, 2008). Once the offspring develop into their larval stage, called glochidia, they are released into the water column and will attach to the gills of a fish host until they develop into juveniles (Texas Parks and Wildlife, 2008). After this stage, they will release from the fish host and fall to the sediments where they will grow to become adults. The average life span for this species is approximately 4-10 years (Texas Parks and Wildlife, 2008).

A. grandis filters large volumes of water and therefore bioaccumulates hydrophobic pollutants found in the water column sorbed to suspended particulates (Gunkel and Streit, 1980). Mussel species are an ideal indicator species due to their quick behavioural and physiological changes in the presence of a contaminant. Behavioural parameters which can be monitored are the opening and closing movements of the valves. Physiologically, the respiration rate of this organism is altered in response to toxic substances. Mussels have been widely used in long-term toxicity experiments.

As they filter and accumulate many chemicals in their visceral mass over their lifespan, sacrificial tests can be conducted to analyse for detrimental levels of chemicals accumulated in the body (Slooff *et al.,* 1983). *A. grandis* has not been heavily used in toxicity experiments for a number of reasons. Firstly, North American toxicity tests using bivalves are not very common. Secondly, there has been a dramatic dynamic change in the mollusca species found in freshwater bodies in the United States and Canada due to many European invasive species such as the common zebra mussel (Johnson *et al.*,

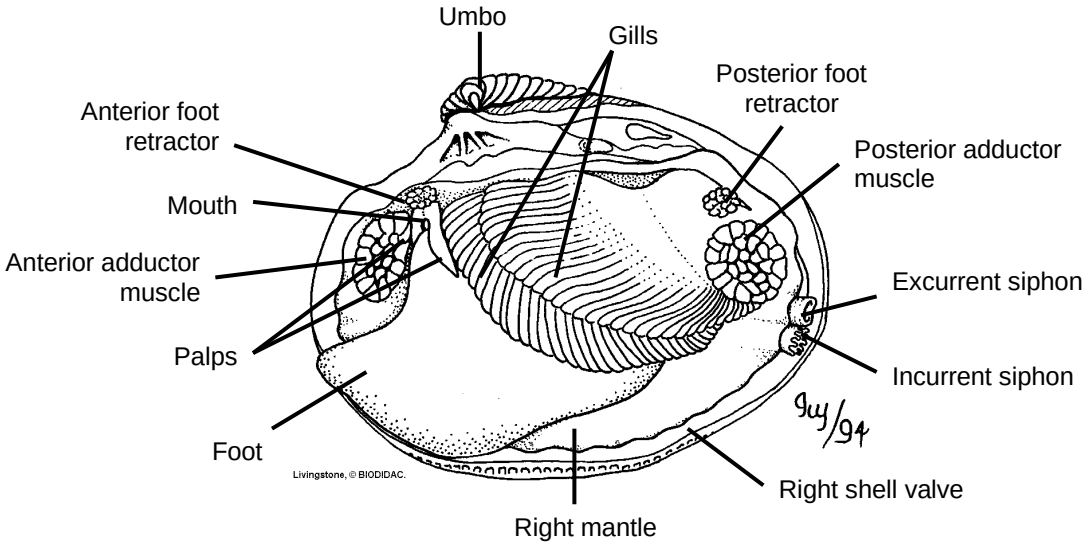


Figure 2.4: Line drawing of the freshwater bivalve *Anodonta grandis* (BIODIDAC, 2009b).

2001). Most of the research conducted in Europe has focused on native species to their specific countries (Borcherding, 2006). *A. grandis* was selected due to its existence as a native species in North American freshwaters and has environmental relevance to toxicity studies in this geographic location.

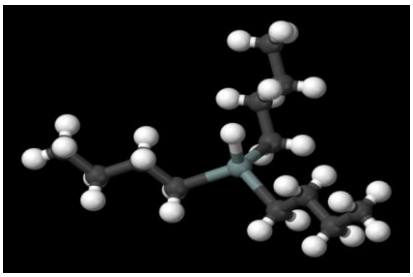
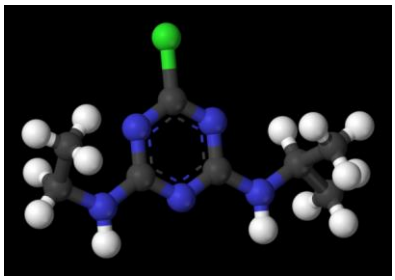
The "Dreissena-Monitor" is a biological early-warning technology developed in Germany which monitors and automates changes in valve movements of zebra mussels (*Dreissena polymorphia*). It is considered to be an ideal early-warning system based on the following criteria: it can operate reliably unattended for a week, it is easy to use and handle, maintenance requirements are approximately 3 hours per week and it contains an automatic alarm system which warns of abnormal behaviours in less than 30 minutes (Borcherding, 2006). The parameters of this system are based on valve movement behaviours and also percentage of open mussels (Borcherding and Volpers, 1994). Zebra mussels are fixated on a flat solid surface and are suspended in a flow-through system in which valve behaviours are detected by a magnet-displacing switch attached to the exterior surface of the shell (Borcherding, 2006). The switch records valve movements under normal conditions and compares these behaviours to stress related conditions with exposure to test chemicals (Borcherding, 2006). Multiple endpoints have already been established by Borcherding (2006) in his ten year experience with this particular model as an early-warning technology. Computer software has been developed for this model which analyses the behavioural movements and produces graphical results (Borcherding, 2006). The Dreissena-monitor is a reliable early-warning system as it is currently being used by thirteen control stations across Germany and is also recommended for use at measuring stations for the "German Commission for the Protection of the Rhine Against Pollution" (Borcherding, 2006).

2.3 Test Chemicals: Tributyltin-hydride and Atrazine

Two test chemicals were selected based on their hazardous effects in the environment and also their ongoing use and persistence in the environment. Tributyltin-hydride (TBT) and atrazine (Table 2.1) are both characterized as pesticides and are currently being used for the control of pests (Chèvre *et al.*, 2006; Luan, 2006; Nikolaou *et*

al., 2007). Experimental concentrations for test chemicals were selected based on their existence in aquatic environments and also with respect to the level of regulation set out by two different government agencies, the USEPA and Health Canada and are outlined below.

Table 2.1: Chemical properties of tributyltin-hydryde and atrazine.

Properties	Trybutyltin-hydride	Atrazine
Ball and stick model		
Chemical structure	$(C_4H_9)_3Sn$	$C_8H_{14}ClN_5$
Chemical group	Tri-alkyl organotin pesticide	Tri-azine herbicide

2.3.1 Tributyltin-hydride (TBT)

Tributyltin-hydride is a tri-alkyl organotin compound which can be found in Canadian freshwaters and can have half-life of 1-2 weeks in aquatic environments (van Slooten and Tarradellas, 1994). The primary mode of degradation is through microbial action and therefore breakdown to its ineffective form may vary with the biota living in the water column. Its presence in Ontario surface waters from international shipping makes it of concern and requires investigation of environmentally relevant concentrations. It has been used heavily since the 1970s and has a multitude of uses such as in: insecticides, pesticides for wood preservation, antifouling agents in paints coating the hulls of large ships and an antifungal agent in textile and industrial water systems (Clark *et al.*, 1988; Dubey and Roy, 2002; Antizar-Ladislao, 2008; Kontrikla, 2009). TBT has been mainly introduced through anthropogenic sources and has been identified as a compound which is harmful to marine and freshwater organisms (Nikolaou *et al.*, 2007). Breakdown of its alkyl groups through degradation either by biotic or abiotic pathways

results in the reduction of its toxicity (Dubey and Roy, 2002). It has been proven to act as an endocrine disruptor mainly affecting reproductive systems and also tends to bioaccumulate within particular organisms (Nikolaou *et al.* 2007). TBT accumulation in gastropods has been shown to act as an endocrine disruptor, resulting in overproduction of testosterone in females, causing imposex characteristics (Antizar-Ladislao, 2008). Imposex causes female gastropods to develop male sex characteristic but continue to obtain female reproductive organs and therefore decrease their overall fitness and a decline in their population (Antizar-Ladislao, 2008). Environmental impacts of TBT causing imposex was in France between 1975 - 1982 where a drastic decline of oyster harvesting occurred and ultimately resulted in a large economic losses (Chiavarini *et al*., 2003). Even though other endocrine disruptors exist in aquatic environments, Chiavarini *et al*. (2003) studies show there is a very high correlation between TBT concentrations and induced imposex characteristics in gastropods. Due to its bioaccumulation properties, this chemical also biomagnifies up the food chain. Yang *et al.*, 2001 have noted that freshwater organisms such as mussels (*Dreissena polymorpha*) have a bioconcentration factor (BCF) of 900,000 and 320,000 for some algae species. Bioconcentration factor is a ratio used in contaminated waters to compae chemical concentrations found within the tissues of an aquatic organism in relation to the surrounding environment (Yang *et al*., 2001).

Bans on organotin additives in antifouling paints have been implemented in many countries primarily on small watercraft since the mid-1980s. The International Maritime Organization (IMO) has called for an international treaty to ban all TBT-containing paints by the beginning of 2008 (Antizar-Ladislao, 2008). Ontario began regulating the use of TBT in antifouling paints in 1989 a complete ban on watercrafts greater than 25-m in length excluding vessels with aluminum hulls but 14 ng Sn/L is still reported in peak months in surface waters (Yang *et al.*, 2001, Yang and Maguire, 2000). Even though these bans exist, TBT has been used for over 40 years and developing countries will continue using these effective and low-cost pesticides (Luan, 2006; Nikolaou *et al.*, 2007). Identifying the toxin in the environment is essential but removal through degradation should be also considered (Luan, 2006). TBT has low water solubility less

than 10 mg/L (water solubility of salt is 357 g/L) and therefore requires a dissolving agent such as ethanol, methanol, acetone or dimethyl-sulfoxide (DMSO) which can be found mixed in freshwaters (Fent, 1996). 0.5% v/v DMSO was selected as a carrier for all TBT experiments due to its negligent effects on the organism's normal behaviour. Some of the above-mentioned carriers might propose detrimental behavioural effects. It is essential to add low concentrations of DMSO to provide a well mixed homogenous system for chemicals such as TBT.

2.3.2 Atrazine

Atrazine is a tri-azine herbicide and is one of the most commonly used pesticides for weed control on agricultural crops in North America (Solomon *et al*., 1996; Chèvre *et al.*, 2006). Its main use is for corn crop protection against broad leaf and grass weeds (Solomon *et al*., 1996). Over 66-million pounds of atrazine was applied on corn crops alone in the US in 2002 which is an 8-million pound increase since 1992 and makes up the majority of the 76-million pound total in 2002 (US Geological Survey, 1992; 2002). In 1994, this organic compound can often be found in freshwaters close to farmlands due to runoff under heavy precipitation conditions (Chèvre *et al.*, 2006). The entire European Union (EU) set a ban on the application of atrazine in 2004 due to the potential contamination of groundwater (Ackerman, 2007). In some parts of Europe such as Switzerland, pesticide application has been banned for over 19 years (Acero, 2000).

This herbicide blocks the electron transport chain between photosystem II (PSII) and photosystem I (PSI) therefore having an effect on the photosynthetic ability of targeted plants (Caux *et al.*, 1996; Fairchild *et al.*, 1998). This chemical is used to control unwanted weeds which only increase crop yields by 6% at most (Ackerman, 2007). Even moderate use of this pesticide facilitates transport into aquatic environments, thereby possibly effecting non-target biota. It has also been considered to be an endocrine disruptor in animals (Brodkin *et al.*, 2007). Its solubility in water is 33 mg/L at 22ºC and has a half life of 13-261 days in soil and 8-14 days in water (Dugay *et al.*, 1998). This low solubility limits the bioavailability of pure atrazine present in freshwaters while the varying half life makes it difficult to determine its persistence in the environment. The

half life of a chemical is determined when it is broken-down to half its initial value and therefore becomes less harmful in its reduced state to non-target organisms.

Environmentally-relevant concentrations of atrazine in the Great Lakes fluctuate around 0.12 µg/L and rarely exceed 20.0 µg/L (Ministry of the Environment Drinking Water Ontario, 2008b; Solomon *et al*., 1996). Non-target organisms could be exposed to concentrations exceeding 20.0 µg/L in the event of storm runoff but this exposure is very transient (Solomon *et al*., 1996). The maximum concentration levels (MCLs) in the United States are 3 µg/L (Acero, 2000, USEPA, 2003b). These levels are slightly higher in Canada, where limiting concentrations of atrazine are 5 µg/L (Health Canada, 1993). 0.5% v/v DMSO was also used as a carrier for atrazine to ensure a perfectly mixed solution. Strong *et al*., (2000) have developed a method to treat atrazine contaminated soils using recombinant *Escherichia coli* which express atrazine-chlorohydrolase which breaks down the hazardous herbicide.

2.4 Early-Warning Biomonitoring Background

A biological approach to determine aquatic toxicity provides an effective and accurate representation in comparison to chemical analysis. It requires the integration of multiple living organisms and the monitoring of their behaviours. Human observations deliver subjective results and therefore the reliance on automatic image analysis and computer software should be used to monitor the behaviours of selected organisms. Construction of a flow-through system is ideal and would require some engineering to design a system to monitor the unique behaviours of individual species

Identifying toxicity in freshwater is an important focus in reducing health risks. With recent concern on bio-terrorism contaminating drinking water sources, it drives the development of a rapid early-warning biomonitoring system to discover lethal contaminants rapidly and to reduce such threats. Chemical tests are expensive, time consuming and do not identify all toxicants present. A biological approach via monitoring the behaviour of multiple organisms would deliver rapid results using sub-acute endpoints, under 24-h, to determine contaminated aquatic systems. Once an alarm is

sounded, due to a significant change in one or multiple organisms' behaviour, a water sample would then be tested chemically to determine the specific responsible for the changed behaviour. Outlined below are possible systems delivering short-term responses which could be used in the development of the multispecies early-warning biomonitoring system being developed in this work.

2.4.1 ECOTOX

ECOTOX is an early-warning biomonitoring technology that was developed in Erlangen, Germany, to signal short term alterations of *Euglena gracilis*' behaviour using an easy-to-use real-time image analysis (Häder, 2007). Tahedl and Häder (1998) have determined specific endpoints for *E. gracilis* such as motility and orientation with this system. The parameters monitored in this system include alignment, upward movement and compactness of this organism prior to and following chemical exposure (Häder, 2007). If a certain parameter threshold is exceeded, an alarm is sounded to notify the operator of unfit biological conditions. Experiments conducted by Tahedl and Häder with the ECOTOX system delivered EC_{50} values which were compared to EC_{50} values using a bioluminescence inhibition assays with *Vibiro fischeri.* This system is fully automatic once all components are set up for use. Complete analysis of one sample can be conducted in less than 10 minutes and therefore it is a representative tool for a early-warning biomonitoring system (Tahedl, and Häder, 2001).

2.4.2 Multispecies Freshwater Biomonitor (MFB)

Another ecotoxicological system used for rapid testing of multiple organisms' behaviours is the Multispecies Freshwater Biomonitor (MFB) (Gerhardt *et al*., 2002). The MFB system has employed a number of animals suspended in the water column and also ones found in sediments such as *C. elegans*, *Daphnia magna*, tadpoles and fish (Gerhardt *et al.*, 2002). These experiments are conducted in a flow-through system and uses quadrapole electrical impedance methods to monitor the changes in electrical current delivered by the test organism (Gerhardt *et al.*, 2003). This system has a free-flow design containing a pair of conducting electrodes on one end and a pair of non-conducting electrodes on the opposite end of the vessel (Gerhardt *et al.*, 2003). A single indicator

organism is placed inside and remains suspended within the test chambers (Gerhardt *et al.*, 2003). Any changes in the electric current between the two sets of electrodes correlate to either the respiration or motility of the indicator species and is recorded in graphical form (Gerhardt *et al.*, 2002). The advantage of using MFB over image analysis tools is that this system can be used for both sediment toxicology and water column tests (Kirkpatrick *et al.*, 2006). Image analysis requires light to capture the organism's behaviour whereas the MFB system monitors changes in electrical current and therefore the MFB system can be employed under dark and even turbid conditions as well as in the light. The MFB system also runs fully automated and computer recognition software has been established for a range of organisms. The complete testing procedure, to determine a change in behaviour, can be accomplished in approximately 5 minutes (Kirkpatrick *et al.*, 2006). MFB has not been used in North American but has been tested in Europe and has proven to accurately detect stressed behaviours of a number of organisms. Therefore, this is another potential system in the detection of environmentally toxic conditions delivering sub-acute results for an early-warning biomonitoring system.

2.4.3 Carbon Dioxide and Oxygen Monitoring

Williams and Jenkinson (1982) indicates that oxygen is the most reliable endpoint measurable for plants and animals because it is either the product or reactant in biological processes. Plants are primary producers and therefore undergo photosynthetic reactions where carbon dioxide is a reactant and oxygen is produced (Tothill and Turner, 1996). Most animals use oxygen to facilitate metabolic reactions and undergo respiration (Tothill and Turner, 1996). These two gases can be quantified to identify abnormal photosynthetic/respiration rates in plants and animals and determine stress responses. Dissolved oxygen probes or a respirometer may be used to measure the concentration of oxygen to indicate how the process of photosynthesis/respiration is altered in the presence of stressors compared to normal conditions (Tothill and Turner, 1996). Depending how quickly different stressors affect the light and dark reactions, it will determine the degree of flux and the location of disturbance in the photosynthetic reaction (Tothill and Turner, 1996). Chen *et al*. (2001) used dissolved oxygen (DO) probes in their

respiration tests with 9 different mussel species to determine fluctuations in oxygen consumption. Winkler titration delivers one of the most accurate measurements for DO in aquatic environments, having a coefficient of variation of only 0.5%, and is favoured over DO probes for accuracy (Williams and Jenkinson, 1982). This technique is quite simple and relatively rapid but very tedious and when titrating a large number of samples could result in diminishing the quality of results (Williams and Jenkinson, 1982). Monitoring and analysing oxygen consumption/development could be used as a parameter in developing an early-warning biomonitoring system for plants and animals.

2.4.4 Integration of Biomonitoring Systems

Integrating the ECOTOX, MFB and CO_2/O_2 monitoring systems to assess the photosynthetic/respiratory and locomotion behaviours of indicator organisms such as, *L. minor*, *P. subcapitata*, *E. gracilis* and *A. grandis* should suffice in rapidly and accurately identifying a large range of contaminants in freshwater sources. Determining additional harmful concentrations of additional chemicals will help expand the list of identifiable toxins which is necessary to optimally use an early-warning biomonitoring system. A limited amount of research has been conducted on early-warning biomonitoring systems, meaning that it is an ideal field to investigate. Once the integration of biological responses (to stressors) have been identified, an engineering solution to extract these contaminants is the next step in preventing unsafe drinking water. Traditional European biological early-warning systems (BEWS) have a very limited scope of information that they provide to end-users. European researchers such as Tahedl and Häder (1998) use a single-organism application which may produce false positives, or more detrimentally, false negatives and they are unable to identify culprit classes of stressors. Gerhardt *et al.* (2003) approached BEWS with a multi-species system but only isolates changes in electrical currents (locomotion and respiration). Our system radically expands upon these simplistic BEWS by incorporating multi-organism, multi-response parameters in models that allow a much more accurate assessment and identification of incoming stressors. Results from this study will directly influence human exposure to chemical contaminants contained in drinking water sources and reduce health risks.

CHAPTER 3

MATERIALS and METHODOLOGY

Each organism was monitored for selected sub-acute parameters outlined below. Each organisms' culturing practices and their specific experimental procedures to monitor normal behaviours in contrast to any changes under the addition of test chemicals over a given time period are outlined below. Concentrations of the two test chemicals TBT (Sigma Aldrich, 234788, 97% purity) and atrazine (Ultra Scientific, CPS-380, 98% purity) were diluted in 0.5% v/v DMSO prior to addition in corresponding test vessels.

3.1 Equipment Disinfection

The selected aquatic indicator species are very sensitive to external chemicals and therefore washing experimental apparatus, especially glassware, is of high importance. Prior to and following any experiments, all glassware equipment which would directly come into contact with any of the organisms was washed with acetone three times and rinsed following every wash. This initial wash, in particular, removed any excess tributyltin residue adhered to the walls of the test chambers. Following the acetone wash, a high grade detergent, Extran organic decontaminating soap (VWR Cat#: CAEX0995-1), was used to scrub off and remove any unwanted chemicals. The vessels were then rinsed with distilled water. Lastly, glassware was acid-washed using 10% v/v hydrochloric acid (HCl) and rinsed with distilled water. If certain equipment needed sterilization it was autoclaved and cooled prior to use.

3. 2 Winkler Titrations

Modified Winkler Titration methods were derived from The Environmental Chemistry of Boston Harbor – IAP 2006 Lab 1: Determination of Dissolved Oxygen. Preparation of five reagents was required prior to toxicity testing. Labelled scintillation vials, filled with no head space, required immediate addition of 50µl manganese chloride

($MnCl_2$) and 50 µl of sodium iodide (NaI). The vial was sealed, inverted to mix the reagents well and allowed to precipitate and flocculate. Samples were inverted a second time and left for 30 minutes until precipitate settled again. Sulphuric acid (H_2SO_4) was added (50 µL) and vials were inverted once again until the precipitate disappeared. Samples were then stored in the dark for later titration. After all the samples were collected, they were diluted to a 100 mL solution with deionized water in a beaker and starch indicator was added. Titration of each sample was conducted using a micropipette. Aliquots of a prepared thiosulfate reagent were added (10 µL) to the diluted samples until the initial dark blue solution became transparent marking the endpoint of the titration and dissolved oxygen was calculated based upon the volume of the titrant added (Appendix B).

3.3 Culturing Indicator Organisms

3.3.1 *Lemna minor*

A culturing tank, a 15-L glass aquarium, was cleaned and rinsed with distilled water prior to the addition of any cultures. Culturing medium, Hunter's medium, containing macronutrients and micronutrients, which are required in large and small quantities respectfully, were added to promote optimum growth (Table 3.1). Four solutions were prepared using the concentrations (g/L) of chemicals outlined in Table 1. When preparing solution #4, the ferric citrate must be added first and heated to dissolve the salt prior to the addition of the remaining reagents. A stock solution was prepared by adding each of the four solutions in accordance with the protocol in Table 1 (right column) and 10 g/L of sucrose was later added (Hunter, 1953). Sterile monocultures of *L. minor* were ordered from Wards^{TM} Natural Science (Item# 86 V 7650). The plants were placed in the culturing vessels for two weeks prior to any testing to optimize the organism's growth patterns. The culturing aquarium was placed in a laminar flow hood equipped with a light bank delivering 90 $\mu E \cdot m^{-2} \cdot s^{-1}$ of light and the temperature was kept at 25 ± 2^{o}C. An Einstein (E) is a unit used in irradiance and is defined as one mole of photons regardless of their frequency. Reduced strength (25% of the initial concentration) growth medium was added to the tank on weekly basis to replenish required growth

nutrients (Hunter, 1953). The stock culture was transferred monthly to newly prepared growth medium monthly in order to maintain a sterile culture.

Table 3.1: Hunter's Medium growth medium used to culture *Lemna minor* (Hunter, 1953).

Solution Number	Compound	Concentration (g/L)	Stock soluton/L of water (mL)
1	KNO_3	100	3
	$Ca(NO_3)_2 \cdot H_2O$	240	
2	$MgSO_4 \cdot 7H_2O$	246	3
3	KH_2PO_4	136	3
4	Ferric Citrate*	1.0	1
	Na_2EDTA	2.90	
	H_3BO_3	1.0	
	$MnSO_4 \cdot H_2O$	0.1	
	$ZnSO_4 \cdot 7H_2O$	1.0	
	$CuSO_4 \cdot 5H_2O$	0.03	
	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.1	

3.3.2 *Pseudokirchneriella subcapitata*

All glassware was washed using the technique described above and the transferring equipment was from sterile packaging. One litre of algae growth medium, Bristol's solution (Table 3.2), was prepared by adding 10 mL of each stock (A – F) into a 1-L volumetric flask and filled with distilled water (940 mL). After 1-L solution was made, one drop of 1% $FeCl_3$ solution was added. Six 250-mL Erlenmeyer flasks were filled with 150 mL of the growth medium and then sterilized in the autoclave for 45 minutes at 15 psi. Preparation of Bristol's solution required six stock solutions individually containing one of the following chemicals: $NaNO_3$, K_2HPO_4, $CaCl_2$, KH_2PO_4, $MgSO_4$, $FeCl_3$ and NaCl. A monoculture of algae (Wards^{TM} Natural Science: Item# 86 V 0620) was stored in a cool area at 10^oC on an agar slant. Each flask was inoculated using aseptic technique by looping algae into the sterile medium. Aluminum foil was loosely fitted on the opening of the flasks to allow for gas exchange and minimize contamination. The culturing vessels were then placed on a shaker (20 rpm) under a light bank (90 $\mu E \cdot m^{-2} \cdot s^{-1}$) on a 16:8-h light/dark cycle and kept at room

temperature 24 ± 2°C. Optimum growth (~5.0 x 10^6 cells / mL) occurred after 7-14 days. These primary cultures were then viewed under a compound microscope to identify if there were alternate organisms growing in the medium. If contamination was not a factor then secondary cultures were prepared from these flasks by aseptically transferring 5 mL of the established primary culture into 250 mL Erlenmeyer flasks containing sterile growth medium.

Table 3.2: Growth Medium for *Pseudokirchneriella subcapitata*. Bristol's Solution was prepared using the Ward's Science protocols. Six 400 mL stock solutions were prepared and stored in the fridge for later growth medium requirements. Stocks contained the chemicals below.

Stock	Substance	Stock Solution (g/L)
A	$NaNO_3$	25.0
B	K_2HPO_4	7.5
C	$CaCl_2$	2.5
D	KH_2PO_4	17.5
E	$MgSO_4$	7.5
F	NaCl	2.5

3.3.3 *Euglena gracilis*

Euglena gracilis was cultured in a similar fashion to that of the algae. All glassware was washed using the technique described above and transferring equipment was sterilized. One litre of *Euglena* growth medium (Wards™ Natural Science: Item# 88 V 5200), Euglena-Gro, was prepared and 150 mL was added into six 250-mL Erlenmeyer flasks. Culturing vessels containing growth medium were sterilized in the autoclave for 45 minutes at 15 psi and were allowed to cool down to room temperature prior to transferring the live organisms. A monoculture of *Euglena* (Wards™ Natural Science: Item# 86 V 2650) was inoculated using aseptic technique by pipetting 5 mL of the concentrated vessel of *Euglena* into the sterile medium. A piece of aluminum foil was loosely fitted on the opening of the flasks to allow for gas exchange and to reduce any contamination. The culturing vessels were then placed on a shaker (20 rpm) under a light bank (90µE m^{-2} s^{-1}) on a 16:8 hour light/dark cycle and kept at room temperature 24 ±2°C. Optimum growth occurred after 7 days. These primary cultures were then viewed under a compound microscope to identify if there were alternate organisms growing in

the medium. If contamination was not a factor then secondary cultures were prepared from these initial culturing vessels. Growth medium was prepared as stated above and 5 mL of the primary cultures was transferred into six new 250-mL Erlenmeyer flasks.

3.3.4 *Anodonta grandis*

Because culturing of *A. grandis* from the veliger stage requires an intermediate host, it was not possible to replicate within our laboratory and therefore adult mussels used in the bioassays were ordered from a local supplier (Wards™ Natural Science: Item# 87 V 4420). A 30-L aquarium was prepared one week prior to the arrival of the mussels to ensure room temperature was reached. Silica sand was placed at the bottom of the tank, 5 cm depth and then the tank was filled three-quarters full with dechlorinated water. An aerator, a simple water filter and a glass cover placed over the tank were set up to provide necessary oxygen reserves and to reduce contamination, respectively. Upon arrival from the supplier, the mussels were immediately set afloat in the culturing tank in their shipping container allowing them to acclimatize to the conditions in the tank. After 6 h, they were slowly released to the sediments and remained in the tank for a 24 h holding period prior to any experimental testing. Mussels were fed pulverized dry algal discs (Wardley® Premium Algae Discs™ Item# 4150). Some water in the holding tank was replaced every two weeks by removing a quarter of the volume and replacing it with dechlorinated water.

3.4 Experimental Set-up

3.4.1 *Lemna minor*

Fifteen 50-mL petri dishes were used as test vessels (Figure 3.1). Five individual plants from a stock, containing two fronds each were sterilely transferred to each of their respected 50-mL petri dishes containing growth medium (reference treatment), medium plus 0.5% v/v DMSO (carrier treatment) or medium plus DMSO plus its respected chemical treatment . Experiments were conducted in triplicate (n=3). Lids were placed on the test vessels to reduce contamination from exposure to falling particles from the air. TBT concentrations selected were 1.0, 10.0 and 100.0 µg/L and atrazine concentrations selected were 5.0, 50.0 and 500.0 µg/L. Increasing concentrations of a selected chemical were prepared and added to their corresponding test vessels. Frond production over a 96 h

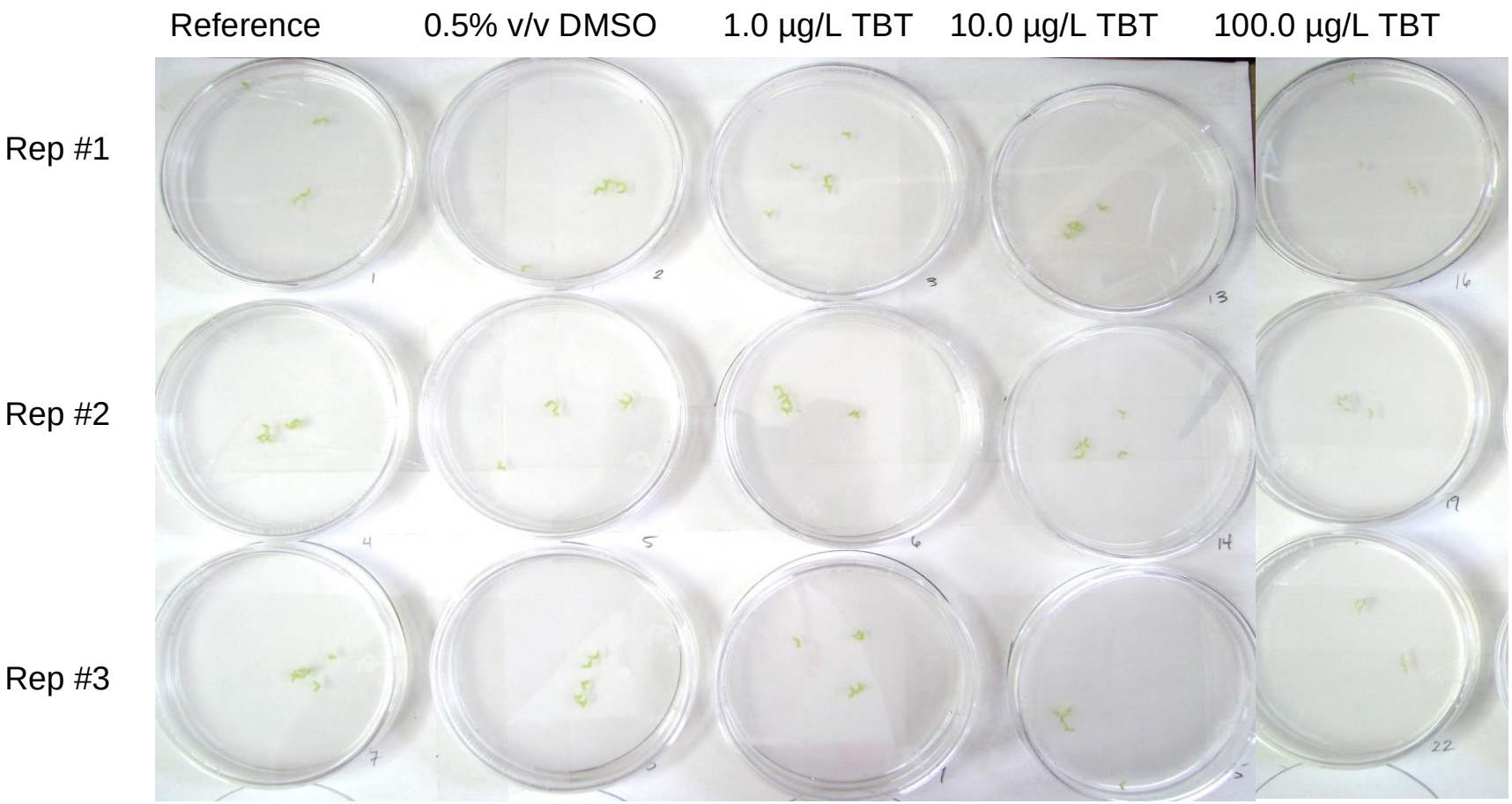


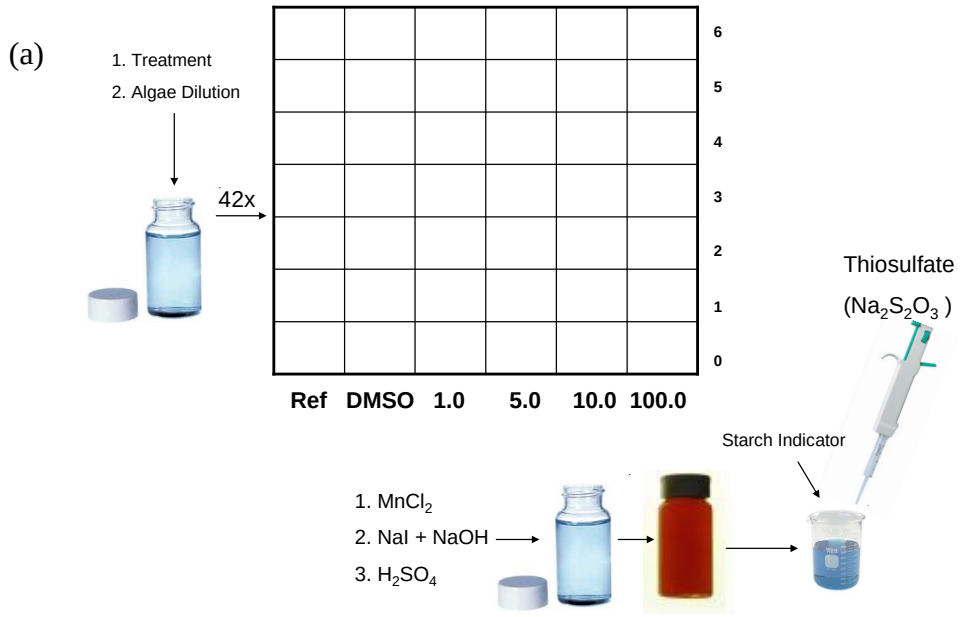
Figure 3.1: Experimental Setup for *Lemna minor.* 15 petri dishes were filled with 30 mL of their respected treatments and randomized. 5 plants containing 2 fronds were placed in each vessel and lids were replaced to reduce contamination and evaporation. Digital photos were taken at sampling times (t=0, 24, 48, 72 and 96h) for the duration of the experiment.

period was monitored and manually counted using time-lapse digital photography in 24 h increments beginning at t=0.

3.4.2 *Pseudokirchneriella subcapitata*

The photosynthetic and growth rates of *P. subcapitata* were quantified via two separate experiments, one which monitored biochemical changes of the photosystems and the other monitored the organism's doubling rate. Results were compared to their normal growth/photosynthetic rate over a defined period of time. Separate experiments were conducted to identify which parameter delivers the best results in minimal time, using the most simplistic methods with minimal costs, yet still deliver rigorous scientific results. Experiments involved measurements of dissolved oxygen (DO) (Figure 3.2). Samples of a known concentration of cells were placed in 25-mL scintillation vials filling the vessels with no head space. These vials were sacrificed every hour for a 6-h period to measure the changes in DO over time. The oxygen content dissolved in the test vessels were accurately measured using Winkler titrations as outlined in Section 3.2. Experiments were preformed in parallel in both the light and the dark environments to compare the effects of the selected chemical on the photosynthetic and respiratory biochemical pathways of the plants. Reference samples were used to contrast the effects of vials exposed to selected contaminants of various concentrations. Another reference treatment was exposed only to the carrier compound, 0.5% v/v DMSO, to identify if this chemical had an effect on the organisms alone. Chemical treatments used in these experiments were 10.0 and 100.0 µg/L of TBT and 50.0 and 500.0 µg/L of atrazine. All experiments were done in triplicate (n=3).

Secondly, the growth inhibition experiments were performed using an Enzyme-linked immunosorbent assay (ELISA) 96-well microplate photometer (MultiSkan-Ascent Thermo Scientific®) to indirectly measure cell counts. Cultures of algae were sub-cultured every two weeks prior to the following experimentation to maintain the organisms in their log growth phase. Microplates (6-well) were used for experimental vessels to maintain a sterile environment (Figure 3.3). As indicated above, a blank (only sterile growth medium), a reference containing only algae (no addition of any chemicals) and 0.5% v/v DMSO treatments were conducted for comparison with different treatments of test chemicals.



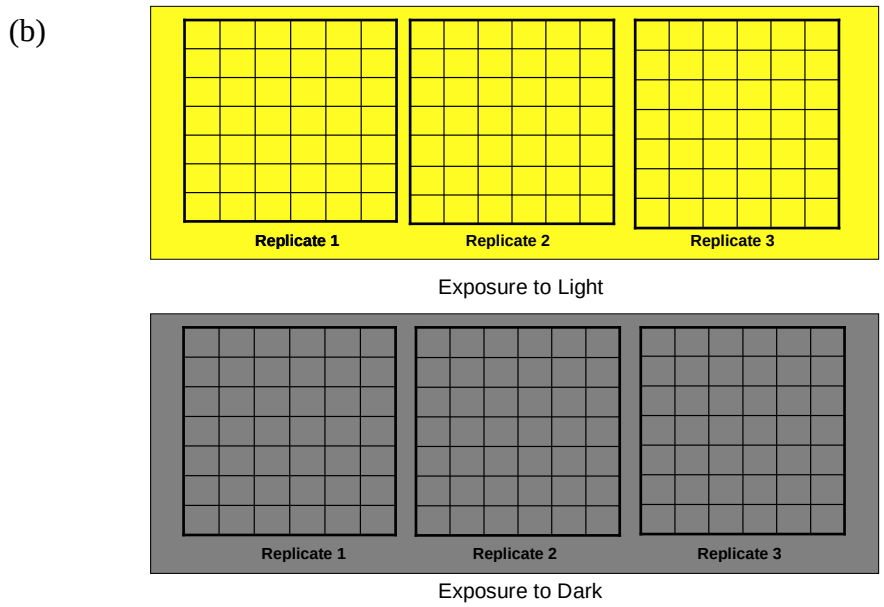


Figure 3.2: Photosynthesis/Respiration Experimental Setup for *Pseudokirchneriella subcapitata* and *Euglena gracilis*. (a) Each scintillation vial is filled (25 mL) with identical dilutions of a selected organism and the corresponding treatment (i.e.: reference, 0.5% v/v DMSO, 1.0, 5.0, 10.0 and 100.0 µg/L of TBT or 5.0, 50.0 and 500.00 µg/L of Atrazine). Each square in the grid below holds a single vial and a total of 42 vials represent one replicate. One vial is sacrificed every hour for the duration of the experiment (6-h total) and Winkler titrations are conducted on individual samples to determine DO content. Treatments are located across the bottom of the rack and time is labelled on the right. (b) Experiments were conducted in triplicates (n=3) in both the light and the dark.

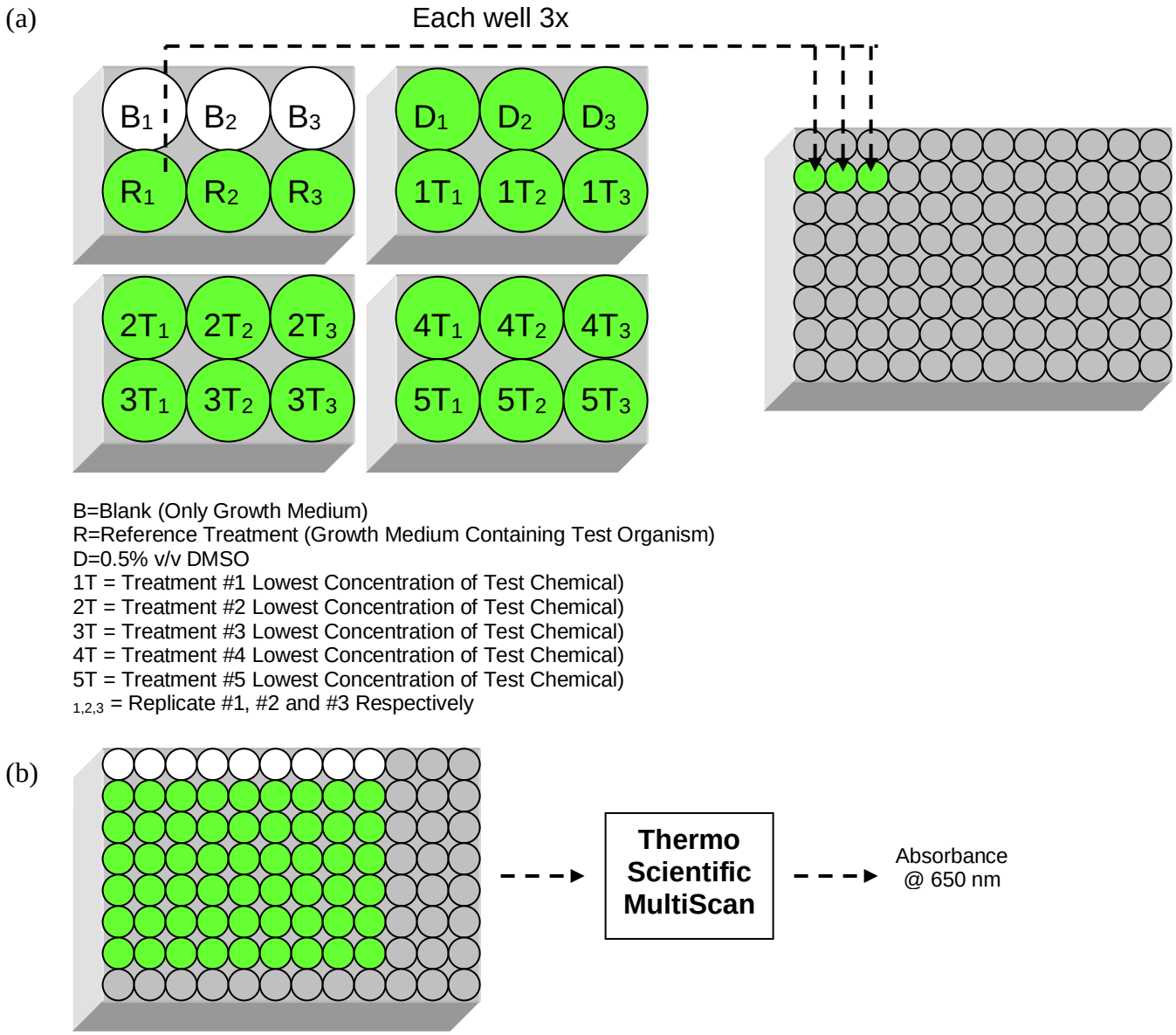


Figure 3.3: Growth Inhibition Experimental Setup for *Pseudokirchneriella subcapitata* and *Euglena gracilis*. (a) 6-well microplates are filled with 7.0 mL of their respected concentration of a selected test chemical. At sampling times (t=0, 3, 6, 12, 24, 48, 72) 200 µL was extracted out of each well then placed in a 96-well microplate and repeated 3 times. (b) Once the necessary wells have been filled, the 96-well plate was placed in the Thermo Scientific MultiScan ELISA reader and absorbance of each well was read at 650 nm.

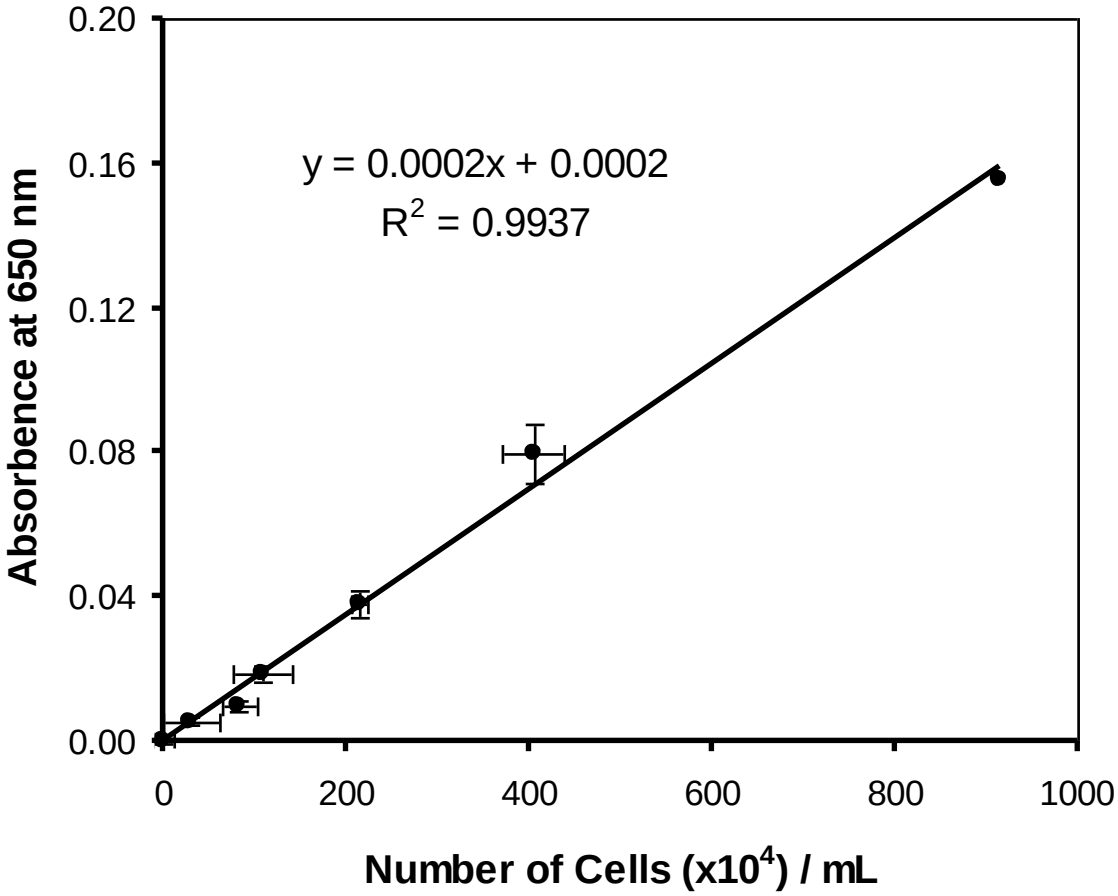


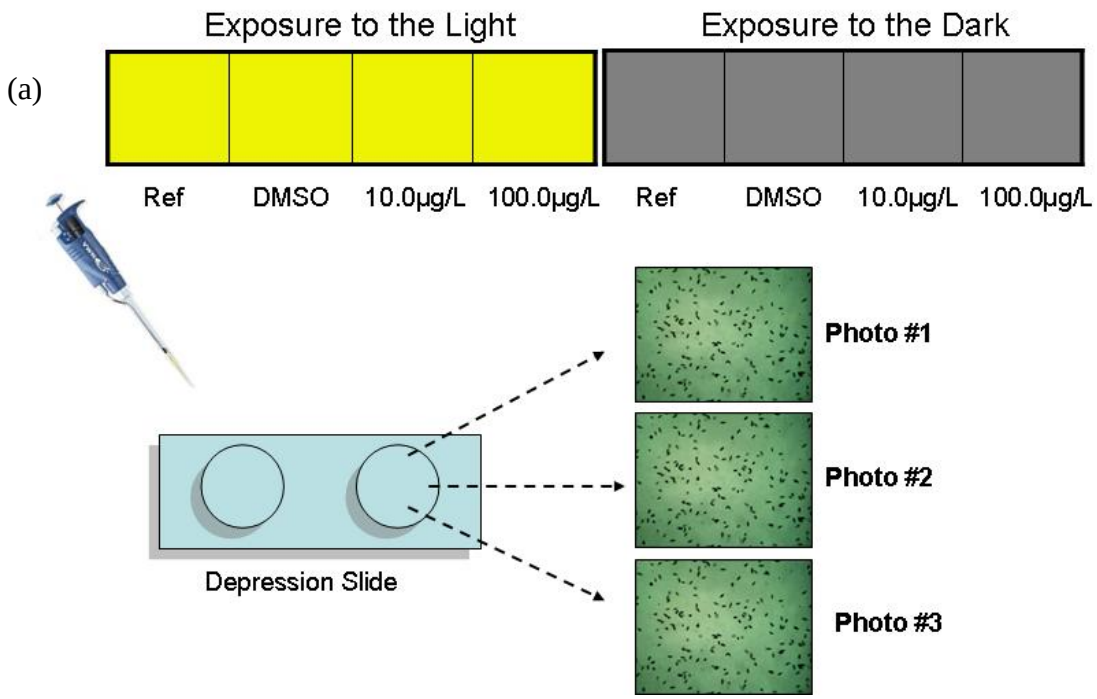
Figure 3.4: Standard regression curve between *Pseudokirchneriella subcapitata* cell counts and OD readings from an ELISA plate reader. X (n=3) and Y (n=7) ± standard error bars and the r^2 are shown.

Measured volumes of chemical treatments were placed in each of the wells and always done in triplicate. Chemical treatments used in these experiments were 1.0, 10.0 and 100.0 µg/L of TBT and 5.0, 50.0 and 500.0 µg/L of atrazine. Algae was then added at time zero and the first reading was taken. 200 µL volumes were extracted in triplicates from each well in the 6-well microplates and transferred into a 96-well microplate. The 96-well plate was placed in the ELISA microplate photometer which determined the absorbance of each well based on the optical density (OD) at 650 nm and, indirectly, the number of cells present in each treatment. A standard regression curve (Figure 3.4) was initially constructed using a known concentration of algae dilutions and correlating these samples with OD readings from the ELISA. Once algae cell dilutions were prepared each test tube was verified using a haemocytometer to count individual cells in a defined volume. Counts were done in triplicate (n=3) and absorbance readings were done seven times (n=7) to deliver a slope of 0.0002 with an r^2 of 0.9937.

3.4.3 *Euglena gracilis*

The stress behaviours of *E. gracilis* can be monitored to deliver information about its surrounding environment. Under stressed conditions the motile organism will alter its shape from its normal elongated spindle form into a contracted stressed cyst conformation (Figure 3.5: (b)). A known concentration of *Euglena* stock was placed in 25-mL scintillation vials and then a set of tests were placed in the two different lighting conditions, light and dark. When *E. gracilis* was exposed to increasing concentrations of a selected chemical, a ratio of spindle shape to cyst form over a 6-h period can be observed. Chemical treatments used in these experiments were 10.0 and 100.0 µg/L of TBT and 50.0 and 500.0 µg/L of atrazine. Digital still photography using a microscope Charged-coupled device (CCD) camera captured the shape change over time. Cell types were counted manually with the help of a computer software program (ImageJ) to label and count the different cell morphologies.

E. gracilis also has plant characteristics and therefore its photosynthetic output can be monitored. Thus, respiration endpoints were utilized. Standardized diluted samples



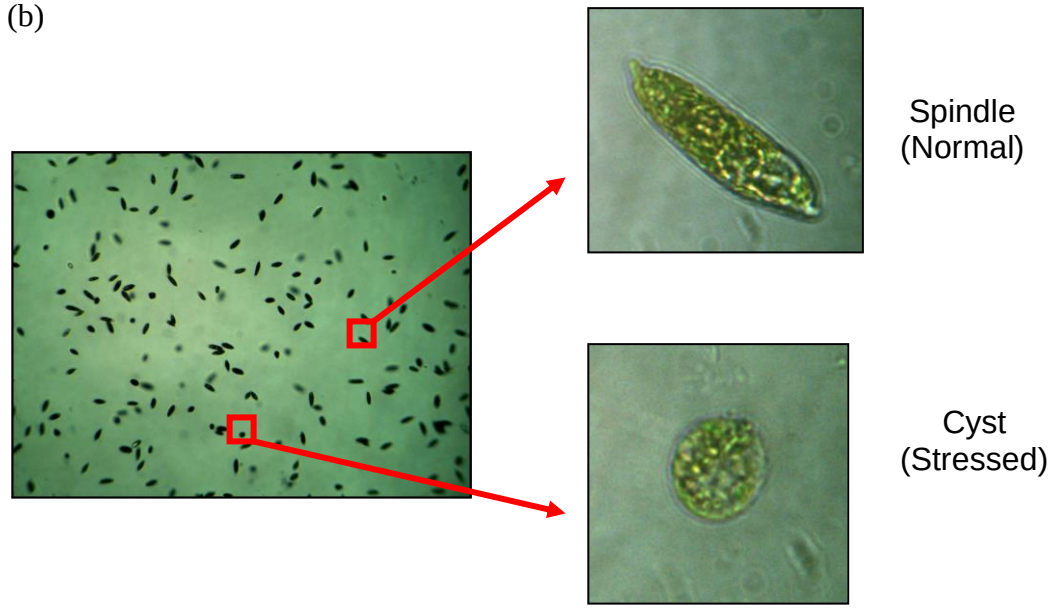


Figure 3.5: Behavioural Experimental Setup for *E. gracilis*. (a) 25 mL scintillation vials were filled with corresponding treatments. At particular sampling times (t=0, 2, 4 and 6h) 20 µL of a given sample was placed on a depression slide and 3 digital photos were randomly taken. (b) Photos were later analysed by manually counting the morphology of the organisms (Cyst or Spindle).

of *E. gracilis* were placed in 25 mL scintillation vials and sacrificed every hour over a 6-h time period (Figure 3.2). Chemical treatments used in these experiments were 10.0 and 100.0 µg/L of TBT and 50.0 and 500.0 µg/L of atrazine. DO was monitored using the Winkler titrations as outlined in Section 3.2 and the production of oxygen in reference vials were compared to vials exposed to increasing concentrations of a selected test chemical.

Growth inhibition experiments were also conducted on *E. gracilis*. 6-well microplates were used for experimental vessels to maintain a sterile environment (Figure 3.3). A blank (only sterile growth medium), a reference containing only *Euglena* (no addition of any chemicals) and 0.5% v/v DMSO treatments were conducted for comparison with different treatments of test chemicals. Measured volumes of chemical treatments were placed in each of the wells and done in triplicate. Chemical treatments used in these experiments were 1.0, 10.0 and 100.0 µg/L of TBT and 5.0, 50.0 and 500.0 µg/L of atrazine. *E. gracilis* was then added at time zero and the first reading was taken. 200 µL volumes were extracted in triplicate from each test vessel and transferred into a 96-well microplate. The 96-well plate was placed in the ELISA microplate photometer which determined the absorbance of each well based on the optical density (OD) at 650 nm and indirectly the number of cells present in each treatment. A regression curve was produced comparing the relationship between cell counts and absorbance at 650 nm (Figure 3.6). Concentrated samples of *E. gracilis* were counted using a haemocytometer (n=4) and then placed in a 96-well microplate photometer (MultiSkan-Ascent Thermo Scientific®) to correlate the number of organisms with a specific absorbance (n=7). This regression curve was used to estimate cell counts and comparisons between reference and test chemicals treatments. Counts were done in triplicate (n=3) and absorbance readings were done seven times (n=7) to deliver a slope of 0.0048 with an r^2 of 0.9986.

3.4.4 *Anodonta grandis*

Prior to experimentation, each mussel was placed in its corresponding 1-L test vessel with 995-mL of dechlorinated water for 12 h. A 5-mL dilution of the test chemical was

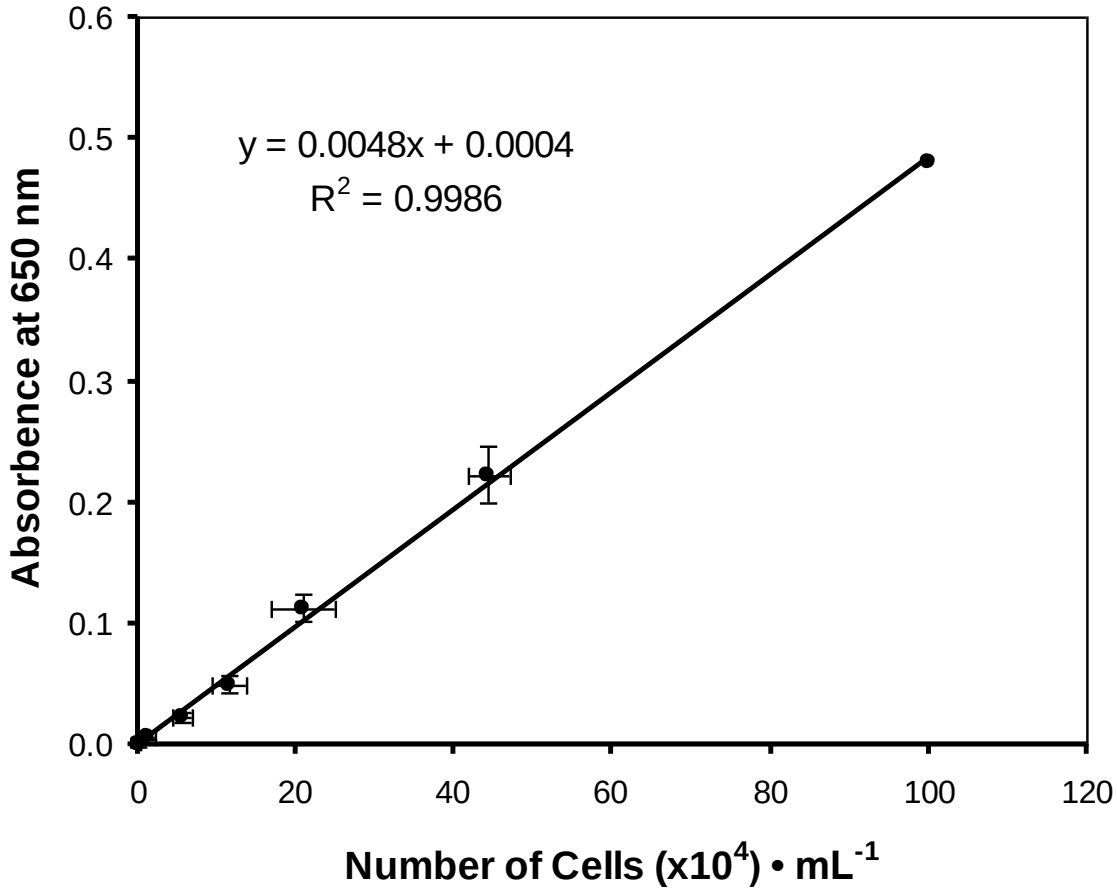


Figure 3.6: Standard regression curve between *Euglena gracilis gracilis* cell counts and OD readings from an ELISA plate reader. X (n=4) and Y (n=7) ± standard error bars and the r^2 are shown.

added to the corresponding 1-L beakers at t=0 and a modified watchglass with a sample extraction tube protruding through the center was placed afloat on the surface of the vessel (Figure 3.7). This modified lid isolated the contents of the test vessel to minimize aeration of the sample and contaminants falling from the air. Chemical treatments used in these experiments were 0.1, 1.0 and 10.0 µg/L of TBT and 0.5, 5.0 and 50.0 µg/L of atrazine. Tests were conducted in five replicates (Figure 3.8).

Multiple endpoints were monitored at one time. While DO samples were being extracted, behavioural observations were also made every hour for a total of 6-h. Behavioural tests were done by observing the valve movements and recorded as either open or closed positions. Respiration rates were conducted by extracting 25 mL samples from the test vessels via the extraction tube. Samples were slowly added to scintillation vials to reduce aeration. The Winkler titration method, outlines in Section 3.2, was performed to determine the DO content of individual samples of TBT. A DO probe (NexSens Smart USB Dissolved Oxygen Sensor, WQ-DO) was used in atrazine due to its ability to determine DO accurately for these particular experiments. This particular DO probe connects to a computer via a USB port and can deliver DO readings in real time. The probe was incorporated in to the experimental design to determine if it delivered similar sensitivity for changes in DO over the course of the experiment. The design of the experiment using the DO probe was the exact same as indicated above with a few modifications, the DO was quantified with the DO probe as oppose to Winkler titrations. The watchglass lid with extraction tube was not required but was replaced with a Parafilm cover to prevent oxygen dissolving into the test medium.

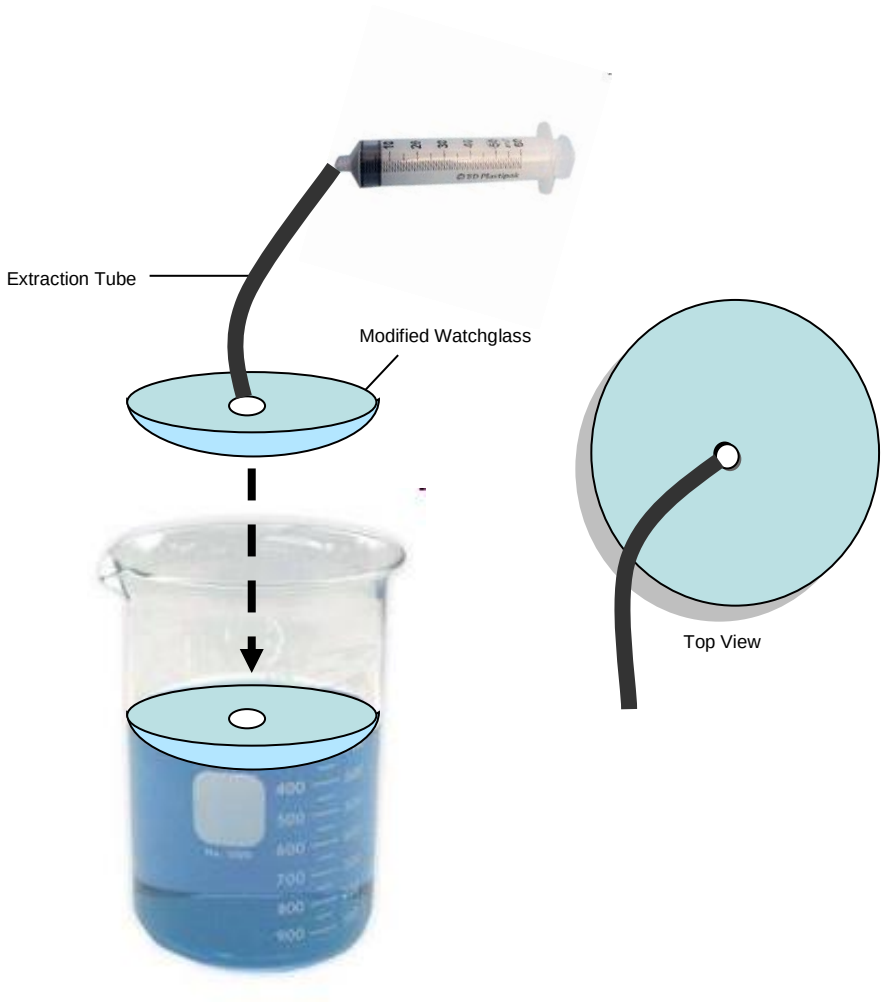


Figure 3.7: Mussel Test Vessel with Modified Lid. A hole was drilled in the center of the watchglass and vinyl tubing was fitted through the opening facilitating sample extraction with a syringe. The modified watchglass was set afloat in the 1-L beaker.

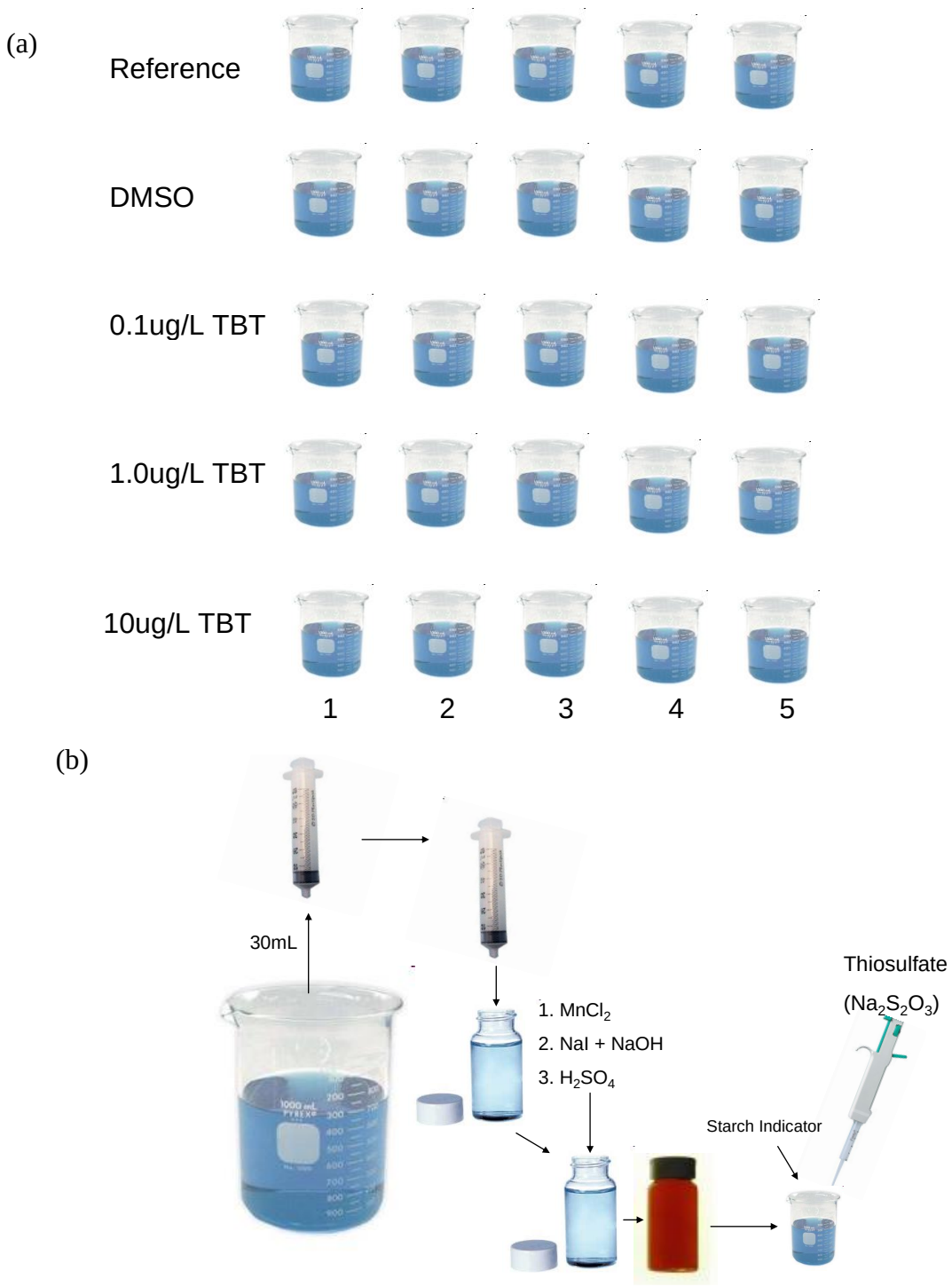


Figure 3.8: Experimental Setup for Freshwater Mussels. (a) 25, 1 L beakers were labelled according to the setup shown above. 5 different treatments, reference, 0.5% v/v of DMSO, 0.1, 1.0 and 10.0µg/L of TBT or 0.5, 5.0 and 50.0 µg/L of atrazine were monitored and samples were extracted every hour for a 6 h period. (b) 30 mL samples were placed in scintillation vials and Winkler titrations were conducted to determine the DO content.

CHAPTER 4
RESULTS and DISCUSSION

Quantities of freshwater sources are not as plentiful as one would think. Protecting the limited quantities that are available is necessary to preserve life (Anderson, 2003; Sabik *et al.*, 2000). Currently, water treatment plants (WTP) chemically treat and test for unwanted chemical and biological contaminants that could cause health risks to end-users. Chemical analysis is an expensive process and takes a considerable amount of time in contrast to a biological approach. This drives environmental studies and specifically this research to develop cost-effective biomonitoring methods, delivering sub-acute, accurate results which enhance current chemical analysis accuracy. The indicator organisms selected for use in the bioassays show sensitivity to selected concentrations of TBT and atrazine.

TBT was used in these experiments to determine if the compound had any effect on the selected indicator organisms. TBT was heavily researched in the past and is banned from use as a paint additive for the shipping industry (Antizar-Ladislao, 2008; Clark *et al.*, 1988; Dubey and Roy, 2002; Kontrikla, 2009). A number of international ships continue to deposit TBT in North American waters due to the lack of implementation of chemical bans in other countries and therefore deserves attention to its effects on non-target organisms. TBT had an effect on all the organisms tested.

Atrazine has been used heavily in the environment on agricultural crops as a commercial herbicide (Chèvre *et al.*, 2006). It is susceptible to runoff to nearby lakes and streams and can even travel to distant freshwaters (Chèvre *et al.*, 2006). Due to its existence in the environment it was selected for investigation on how it affects non-targeted indicator organisms. Experiments were conducted with the same organisms as the TBT bioassays to identify if atrazine had a significant altering effect on the normal behaviours of these species. Changes in the biochemical, morphological, and

physiological parameters due to exposure with atrazine and TBT were monitored and are presented below. A summary of the results are presented in Section 4.3, highlighting the parameters delivering sub-acute responses to atrazine and TBT. These responses deliver support for the inclusion of these organisms in the development of an early-warning biomonitoring system due to their rapid responses in identifying unfavourable aquatic conditions.

4.1 Bioassay organism responses to Tributyltin-hydride (TBT)

4.1.1 *Lemna minor*

Experiments was conducted using *Lemna minor* in conjunction with TBT and investigated the changes to the organism's growth rate over a 96-h test period. Figure 4.1 outlines the results obtained from the growth inhibition experiments showing frond counts at different times (Appendix C). The reference treatment displays the normal growth of fronds and was compared to chemical treatments.

In Figure 4.1, the reference, dimethyl-sulfoxide (DMSO) and 1.0 µg/L treatments were observed to have similar number of fronds at corresponding time intervals. The carrier, 0.5% v/v DMSO, was used to see if it had an effect on the frond production of this plant. DMSO portrayed a similar growth pattern throughout the entire experiment. The development of new fronds in the 10.0 µg/L treatment plateaued after a 24-h exposure and maintained this impeded growth for the remainder of the 96-h experiment. Exposing the aquatic plant to 100.0 µg/L of TBT dramatically halted its ability to produce new fronds. After 48 hours the loss of pigmentation within the majority of the leaves was observed. At the 96-h sampling period, green fronds were undetectable and had complete loss of all chlorophyll. Growth rates were determined for each treatment and statistically analysed (Table 4.1) to determine if there was a significant difference of growth over the experimental period. Significant differences of the growth rate between the reference and all other treatments were analysed using Analysis of Variance (ANOVA) with a Tukey pair-wise *post hoc* comparison to determine the probability that there was a difference between growth rates.

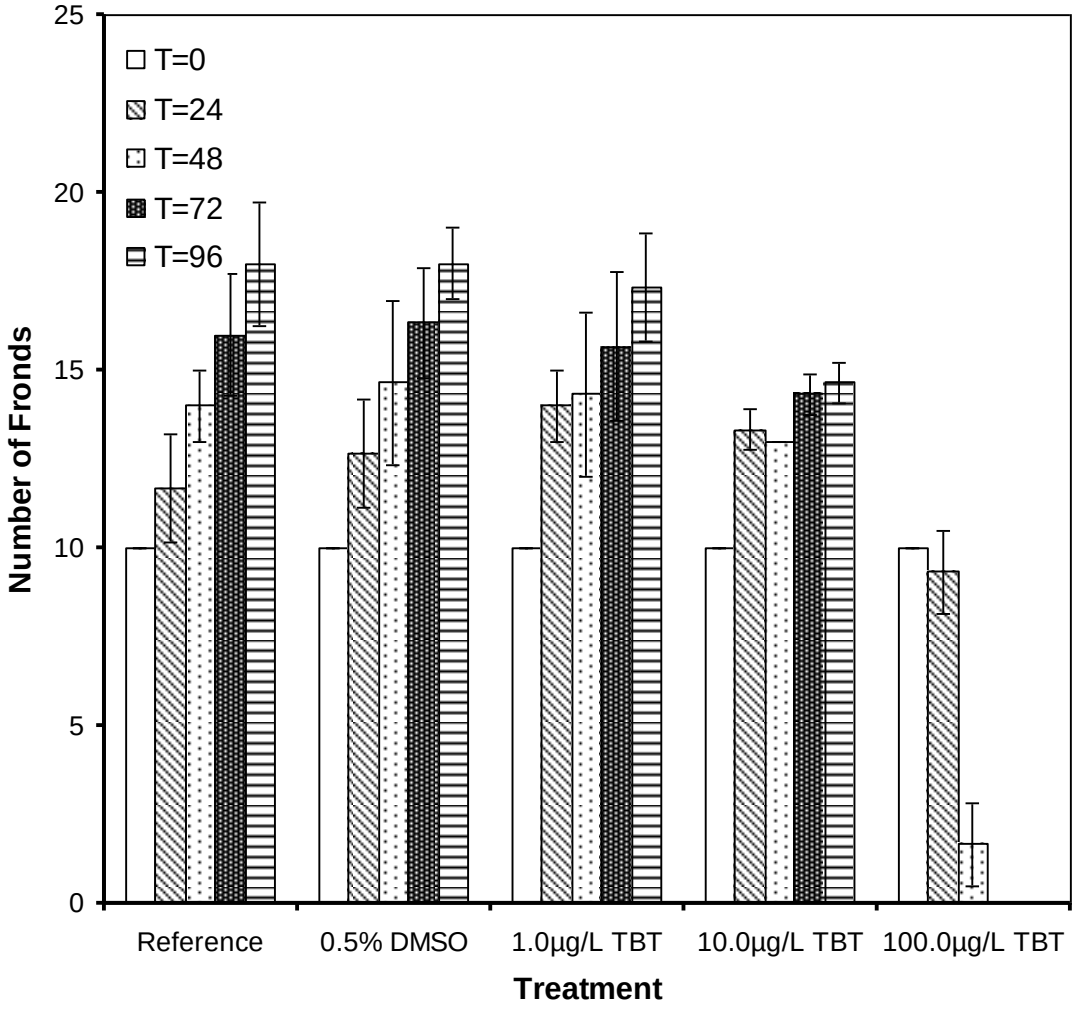


Figure 4.1: Frond counts for *Lemna minor* over a 96-h (T=time (h)) exposure period to three different concentrations of TBT (±SD).

Table 4.1: ANOVA of *Lemna minor* growth rate over 96-h comparing the reference to all other treatments of TBT.

P-value				
Overall	0.5% DMSO	1.0µg/L TBT	10.0µg/L TBT	100.0µg/L TBT
<0.001*	0.925	0.368	0.022*	<0.001*

*Statistically significant values

Table 4.1 outlines the p-values comparing the reference to each treatment. Values less than 0.05 indicated a significant difference between the growth rates. When the growth rates were analysed for variance from the reference treatment, plants exposed to 10.0 and 100.0 µg/L of TBT produced p-values of less than 0.05. This represented a 95% probability that these growth rates were different in comparison to those of the reference test. This test showed that *L. minor* showed a significant declining rate of growth when exposed to concentrations of TBT as low as 10 µg/L. The emergent macrophyte is highly sensitive to relatively higher concentrations of TBT, 100 µg/L, and would deliver early-warning detection of TBT contaminated waters. Therefore, *L. minor* would be a good candidate in the development of a multispecies early-warning biomonitoring system.

4.1.2 *Pseudokirchneriella subcapitata*

Three different bioassays were conducted with *Pseudokirchneriella subcapitata*. The first two tests, photosynthesis and respiration rate, investigated biochemical endpoints where dissolved oxygen (DO) concentrations were quantified using the Winkler titration method in individual scintillation vials. The DO content was then plotted over time to produce the photosynthetic/respiration rate (slope). The final test involved monitoring cell counts over a 72-h period with exposure to different concentrations of TBT.

A) Biochemical Endpoint #1: Photosynthetic Rate (Light experiments)

The photosynthetic rates (slopes) from Table 4.2 (Appendix D) are shown in Figures 4.2. Light experiments were conducted to investigate the fluctuations of DO

development facilitated by the photosynthetic capability of *Pseudokirchneriella subcapitata* and determine if TBT affects the production of oxygen.

Table 4.2: DO content (µmol $O_2 \cdot L^{-1}$) for light experiments with exposure to two concentrations of TBT. Replicates (n=3) were averaged in corresponding sampling periods (± standard deviation (SD)). The slope (µmol $O_2 \cdot L^{-1} \cdot h^{-1}$) represents the photosynthetic rate of the algae over the 6-h experiment.

Time(h)	Average DO Content (µmol O_2/ L) per Treatment (n=3)			
	Reference (±SD)	0.5% v/v DMSO (±SD)	10 µg/L TBT (± SD)	100 µg/L TBT (± SD)
0	0.2229 (0.0032)	0.2208 (0.0050)	0.2229 (0.0018)	0.2234 (0.0041)
1	0.2347 (0.0063)	0.2337 (0.0009)	0.2327 (0.0054)	0.2265 (0.0031)
2	0.2415 (0.0032)	0.2389 (0.0016)	0.2384 (0.0065)	0.2316 (0.0047)
3	0.2430 (0.0054)	0.2441 (0.0024)	0.2425 (0.0065)	0.2322 (0.0009)
4	0.2347 (0.0078)	0.2373 (0.0041)	0.2384 (0.0039)	0.2270 (0.0050)
5	0.2430 (0.0050)	0.2389 (0.0027)	0.2410 (0.0036)	0.2301 (0.0024)
6	0.2575 (0.0047)	0.2575 (0.0047)	0.2534 (0.0059)	0.2368 (0.0039)
Slope	**0.0041**	**0.0042**	**0.0039**	**0.0015**

Figure 4.2 shows that there were similar photosynthetic trends observed in the reference, 0.5% v/v DMSO and 10.0 µg/L of TBT treatment. Light samples of algae exposed to 100.0 µg/L of TBT showed a reduced photosynthetic rate trend in comparison to those of the reference treatment. The photosynthetic rates were also analysed statistically to determine if there was a significant difference between the reference and all other treatments (Table 4.3). Light experiments involving photosynthetic rate were performed using individual isolated vessels and were analysed by determining the equality of two correlations (Kruskal-Wallis test) comparing the reference photosynthetic rates with other three treatments. This statistical test produced p-values (Table 4.3) which indicated if there was a linear correlation amongst the reference and test treatments. The test indicated that there was no significant difference amongst treatments in comparison to the reference. With exposure to the highest concentration of TBT (100 µg/L), it showed to have a declining production of O_2 and therefore this endpoint should be considered as an early-warning indicator.

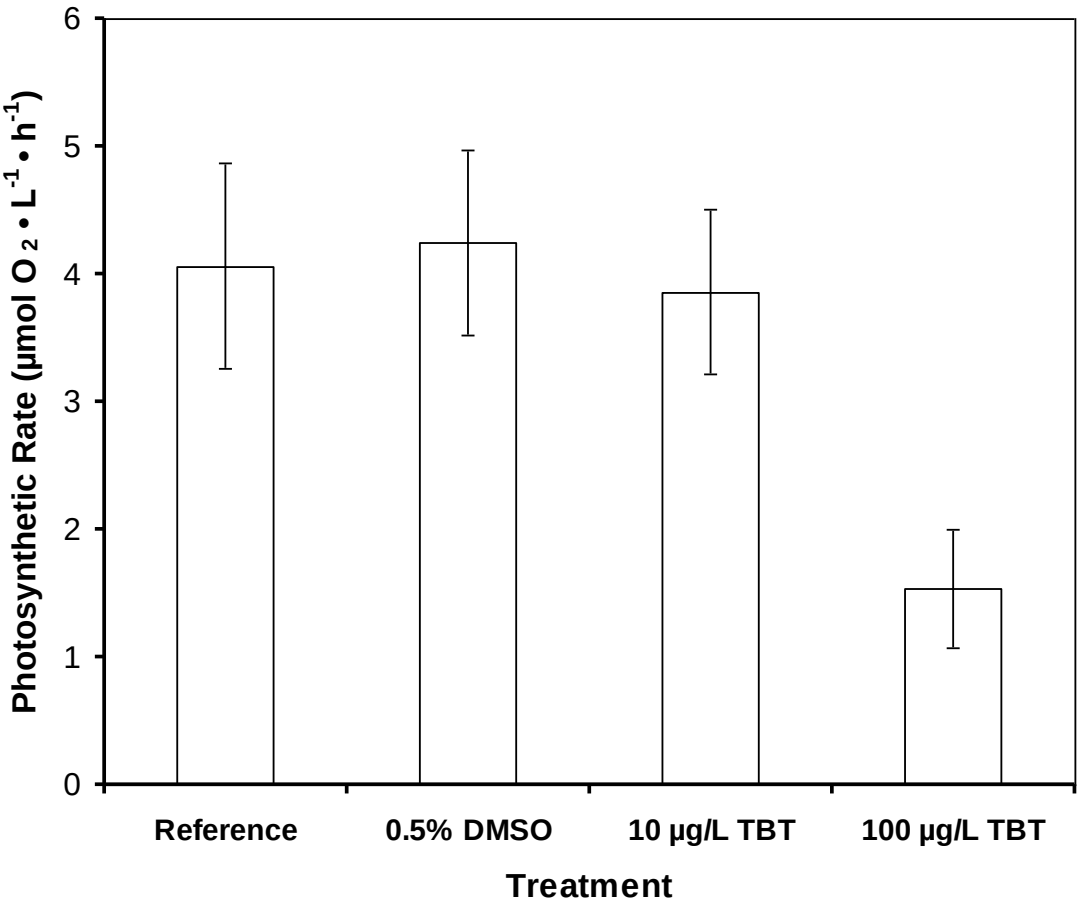


Figure14.2: Photosynthetic rate (µmol $O_2 \cdot L^{-1} \cdot h^{-1}$) ±SD of *Pseudokirchneriella subcapitata* exposed to two concentrations of TBT derived from 6-h light exposure experiments (n=3). Rates were determined from data presented in Table 4.2.

Table 4.3: Comparison between the normal photosynthetic rate of *Pseudokirchneriella subcapitata* to samples exposed to DMSO and TBT. Rates were analysed using the Kruskal-Wallis test which determined the equality of two correlations.

P-value		
0.5% DMSO	10.0µg/L TBT	100.0µg/L TBT
0.722	0.698	0.377

B) Biochemical Endpoint #2: Respiration Rate (Dark experiments)

The second biochemical endpoint investigated the respiration rate of the algae and it was conducted in the absence of light. These sets of experiments were done in parallel with the light experiments to ensure similar environmental conditions. Table 4.4 presents the individual DO measurements (±SD) at each time increment and also the respiration rate (slope) developed by each treatment (Appendix E). Declining O_2 concentrations from respiration delivers a negative slope and therefore the slope was multiplied by negative one to give positive values. The slopes were also transposed into Figure 4.3 which shows the respiration rate for each treatment with its corresponding standard deviation.

Table 4.4: DO content (µmol O_2/L) for dark experiments with exposure to two concentrations of TBT. Replicates (n=3) were averaged in corresponding sampling periods (±SD). The slope (µmol $O_2 \cdot L^{-1} \cdot h^{-1}$) represents the respirations rate of the algae over the 6-h experiment.

Time (h)	Average DO Content (µmol O_2/ L) per Treatment (n=3)							
	Reference (± SD)		0.5% v/v DMSO (± SD)		10 µg/L TBT (±SD)		100 µg/L TBT (± SD)	
0	0.2198	(0.0024)	0.2182	(0.0065)	0.2208	(0.0024)	0.2146	(0.0009)
1	0.2151	(0.0063)	0.2182	(0.0054)	0.2141	(0.0016)	0.2192	(0.0009)
2	0.2161	(0.0024)	0.2192	(0.0024)	0.2161	(0.0018)	0.2151	(0.0009)
3	0.2115	(0.0045)	0.2141	(0.0047)	0.2130	(0.0059)	0.2099	(0.0039)
4	0.2058	(0.0047)	0.2058	(0.0036)	0.2037	(0.0036)	0.2073	(0.0032)
5	0.2073	(0.0039)	0.2068	(0.0018)	0.2068	(0.0024)	0.2084	(0.0024)
6	0.2073	(0.0032)	0.2084	(0.0024)	0.2094	(0.0041)	0.2089	(0.0047)
Slope	**0.0023**		**0.0023**		**0.0022**		**0.0017**	

In the dark experiments with exposure to the carrier, DMSO and two treatments of TBT showed no physiological effect to this particular organism. The oxygen content for each treatment remained the same for the duration of the 6-h experiment (Figure 4.3).

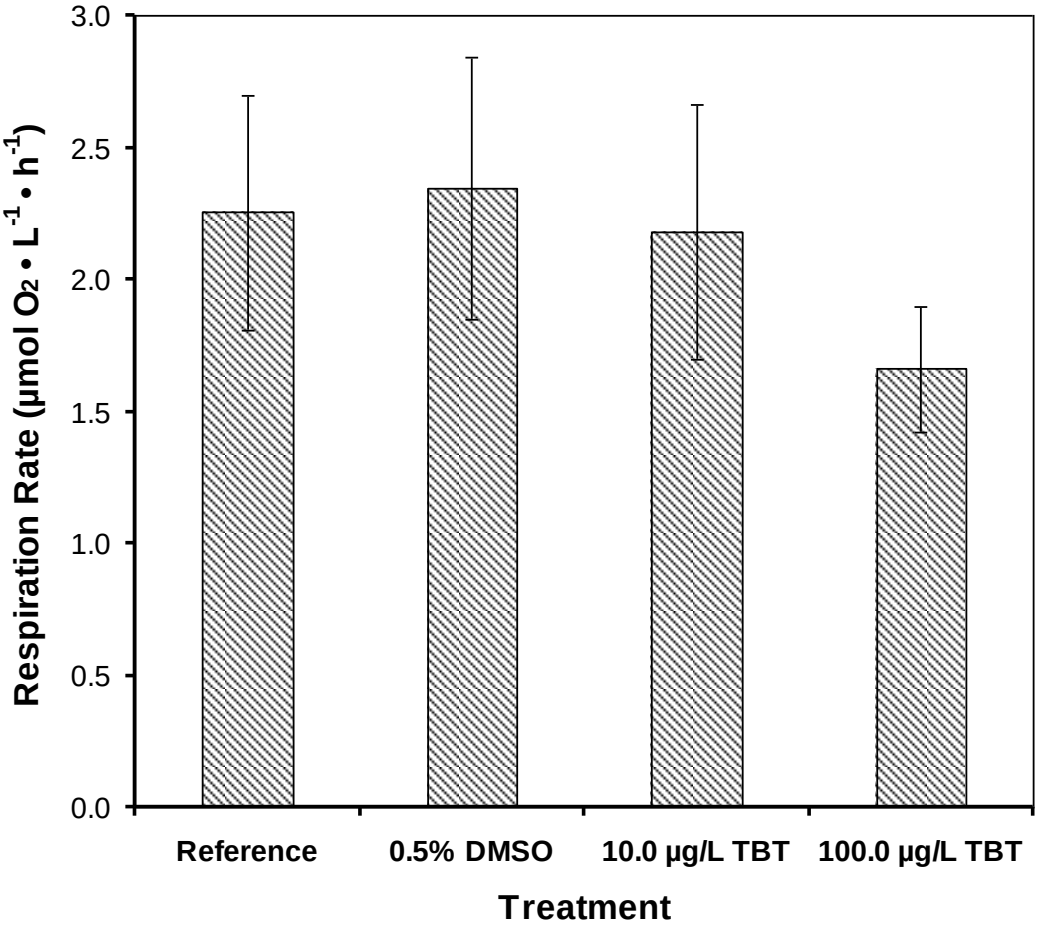


Figure 4.3: Respiration rate (µmol $O_2 \cdot L^{-1} \cdot h^{-1}$) ±SD of *Pseudokirchneriella subcapitata* exposed to two concentrations of TBT derived from 6-h dark exposure experiments. Rates were determined from data presented in Table 4.4.

Further investigation for this experiment was done through statistical analysis using the Kruskal-Wallis test. This statistical test produced p-values (Table 4.5) which indicated if there was a correlation amongst the reference and test treatments. None of the values exceeded 0.05, which gives the data significance. Monitoring the respiration rate of the green algae did not produce significant results and was not an ideal parameter for this particular organism to detect a contaminated environment.

Table 4.5: Comparison between the normal respiration rate of *Pseudokirchneriella subcapitata* to samples exposed to DMSO and TBT. Rates were using the Kruskal-Wallis test to determine the equality of two correlations.

P-value		
0.5% DMSO	10.0µg/L TBT	100.0µg/L TBT
0.877	0.812	0.148

C) Growth inhibition test (Cell counts)

The last endpoint investigated for *Pseudokirchneriella subcapitata* was to observe changes in cell growth over a 72-h period. In photosynthetic plants, a block along the electron transport chain causes interference in normal growth and inhibits the organism's ability to reproduce effectively. Figure 4.4 displays the algae's growth at increasing time increments for all treatments (Appendix F). Cell counts were calculated indirectly using a regression curve (Figure 3.4) which accurately compared cell numbers to OD at 650 nm. It was observed that the carrier, 0.5% v/v DMSO, had an effect on the growth rate for this particular set of experiments which could be due to a contaminated chemical stock of DMSO. Table 4.6 was produced to statistically show that the carrier did have an effect. Also, Table 4.7 was constructed to isolate the effects of the test chemical and not the compound effects caused by the carrier and the TBT. Exposure to 1.0 and 10.0 µg/L of TBT did not seem to have a toxic effect trend. When the algae were treated with 100.0 µg/L of TBT, it showed a quick halt in its growth rate after 24 hours and slow increase of cell death for the remainder of the experiment.

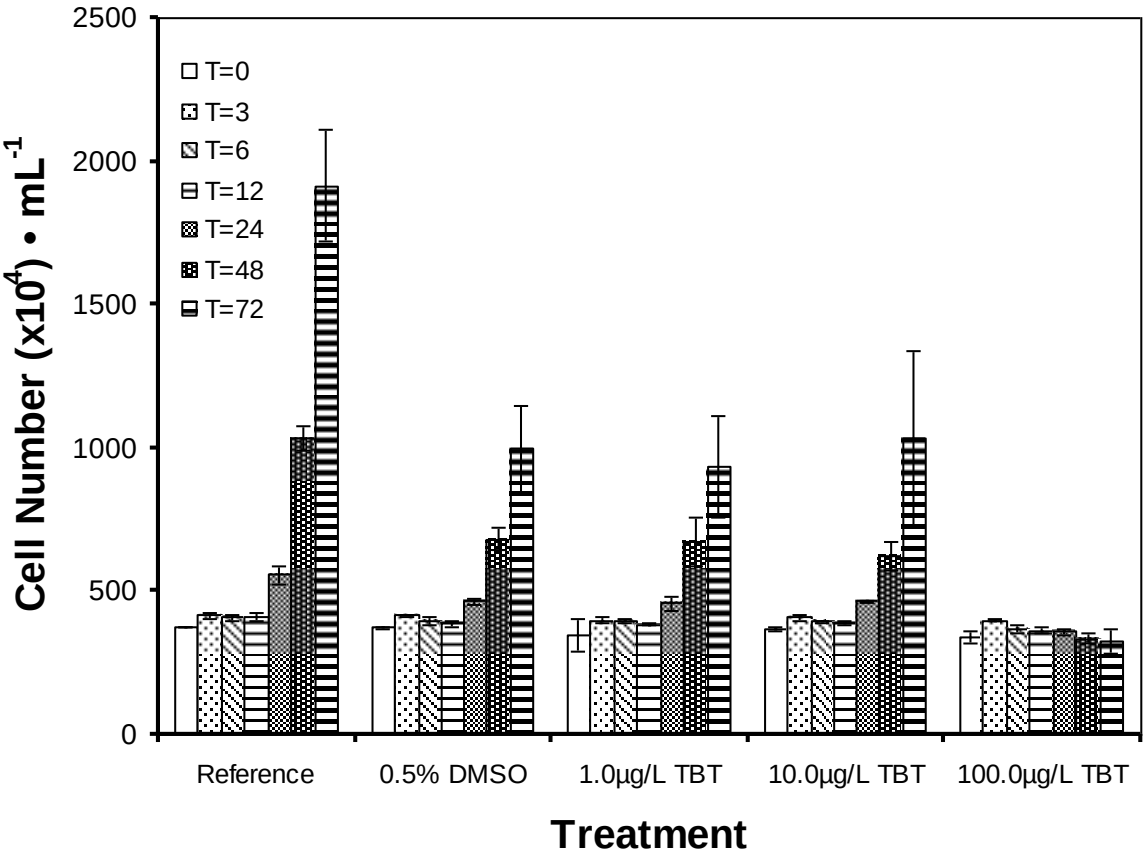


Figure 4.4: Cell counts for *Pseudokirchneriella subcapitata* ±SD over a 72-h (T=time (h)) exposure period to three different concentrations of TBT (n=3). Data was produced using a regression curve (Figure 8) comparing cell counts to OD at 650 nm.

Statistical significance for these growth inhibition experiments was determined using ANOVA with a Tukey pair-wise *post hoc* comparison. The statistical test was used to determine whether selected concentrations of TBT affected the growth rate in comparison to those of the reference treatment. Table 4.6 compared reference growth rates with all other treatments and Table 4.7 compared DMSO exposure with all other TBT treatments. Values less than 0.05 indicated there was a significant difference with 95% probability that there was a difference in growth rate. When comparing all treatments with the reference, there was more than 99% probability that all other treatments had an effect. Therefore, a second analysis was prepared (Table 4.7) comparing effects of only the test chemical. In this table, only the 100.0 µg/L of TBT produced a value of less than 0.05. This test proves that this parameter has potential in delivering sub-acute response when exposed to concentrations higher than 100 µg/L of TBT and therefore should be considered for the early-warning biomonitoring system.

Table 4.6: Statistical analysis of the growth rate of *Pseudokirchneriella subcapitata* over a 72-h period comparing the reference to all other treatments of TBT. ANOVA was conducted on log transformed growth rates.

	P-value			
Overall	0.5% DMSO	1.0µg/L TBT	10.0µg/L TBT	100.0µg/L TBT
<0.001*	0.006*	0.006*	0.006*	<0.001*

*Statistically significant values

Table 4.7: Statistical analysis of cell growth rate for *Pseudokirchneriella subcapitata* over a 72-h period comparing the 0.5% v/v DMSO treatment to all other treatments of TBT. ANOVA was conducted on log transformed growth rates.

	P-value		
Overall	1.0µg/L TBT	10.0µg/L TBT	100.0µg/L TBT
<0.001*	1.000	1.000	0.001*

*Statistically significant values

After three separate experiments, it was determined that *P. subcapitata* responded well as an early-warning bioindicator. Two of the three experiments, photosynthetic rate and cell inhibition tests, are recommended for further experimentation with alternate chemicals to determine the sensitivity at the sub-acute level.

4.1.3 *Euglena gracilis*

Tests performed with *Euglena gracilis* with exposure to TBT were similar to that of *Pseudokirchneriella subcapitata*. In addition to the biochemical tests and the cell counts, *Euglena* also underwent behavioural tests to determine changes in the cell morphology with exposure to TBT. These behavioural experiments were conducted in parallel in both the light and dark environments.

A) Biochemical Endpoint #1: Respiration Rate (Light experiments)

Firstly, *Euglena* underwent biochemical experiments in the light (Appendix G) due to the protist's ability to act as a primary producer and capture light via their internal chloroplasts. Results from the light exposure experiment did not fit a linear relationship and therefore a figure was not prepared. Table 4.8 displays the non-linear relationship by displaying low r^2 values derived from the slope for each treatment. It was determined that this experimental set-up did not allow for proper determination that the organism was experiencing stressful conditions Therefore, *Euglena* respiration rate experiments are not recommended for future experimentation for the purposes of early-warning detection system.

Table 4.8: Respiration rate (µmol $O_2 \cdot L^{-1} \cdot h^{-1}$) of *Euglena gracilis* exposed to two concentrations of TBT derived from 6-h light exposure experiments.

Treatment	Slope (µmol $O_2 \cdot L^{-1} \cdot h^{-1}$)	r^2 value
Reference	-1.3850	0.1491
0.5% DMSO	-1.1265	0.0456
10.0µg/L TBT	-0.0923	0.0010
100.0µg/L TBT	-0.3878	0.0281

B) Biochemical Endpoint #2: Respiration Rate (Dark experiments)

Euglena's ability to undergo photosynthesis and respiration posed conflicting measurements when exposed to light and therefore biochemical monitoring experiments were also conducted in the dark to eliminate the photosynthetic abilities of this organism and isolate their respiratory parameters (Appendix H). Figure 4.5 displays the respiration

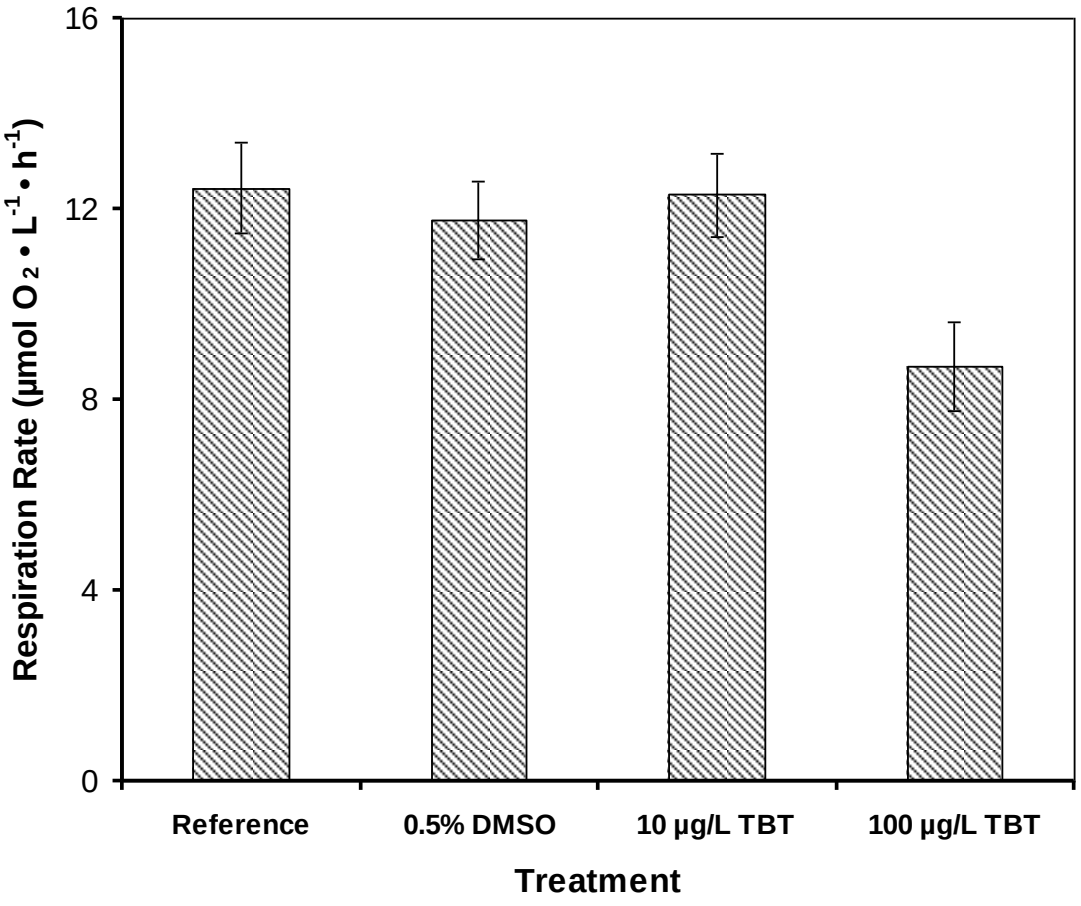


Figure 4.5: Respiration rate (µmol $O_2 \cdot L^{-1} \cdot h^{-1}$) ±SD of *Euglena gracilis* exposed to two concentrations of TBT derived from 6-h dark exposure experiments.

rates (±SD) of E*uglena gracilis* when tests were conducted in the dark. In comparison to the reference treatment, the carrier DMSO shows a similar respiratory trend. Also both treatments of TBT, 10.0 and 100.0 µg/L, do not differentiate very much from the average respiration rate of the reference trails. Respiration rates were analysed using the Kruskal-Wallis test to statistically determine if there was a correlations of the respiration rates when comparing the reference to all other treatments. Values less than 0.05 indicate a 95% probability that the treatment respiration rates are different from the reference. Table 4.9 illustrates there was no significant similarity between the reference treatment by comparing the equality of two correlations. Results from this experiment show that monitoring the respiration rate of *Euglena* was not a useful measurement allowing for early detection of TBT in the water column. Therefore it is not suitable indicator for this specific chemical but could show sensitivity to other aquatic contaminants.

Table 4.9: Comparison between the normal respiration rates of *Euglena gracilis* to samples exposed to DMSO and TBT. Rates were analysed using the Kruskal-Wallis test which determined the equality of two correlations.

	P-value	
0.5% DMSO	10.0µg/L TBT	100.0µg/L TBT
0.736	0.782	0.402

C) Growth inhibition test (Cell counts)

Cell counts were also performed with *Euglena* similar to that with algae (Appendix I). Tests were conducted over a 72-h period and absorbance readings were transformed using a regression curve (Figure 3.6). Figure 4.6 displays the cell counts over a 72-h period for all treatments. Data plotted in Figure 4.6 was log-transformed prior to ANOVA testing with a Tukey *post hoc* comparison. The log transformation was performed to allow linear analysis of the data. Once the data was transformed, a slope was calculated for cell numbers over time. This slope was then analysed using an ANOVA test to determine if the reference growth rates differed in comparison to the TBT-treated tests. Figure 4.6 indicates that there is no growth rate change for any of the TBT treatments. Statistical analysis of the cell counts was performed to determine if the TBT treatments had a significant correlation with the reference as shown in Table 4.10.

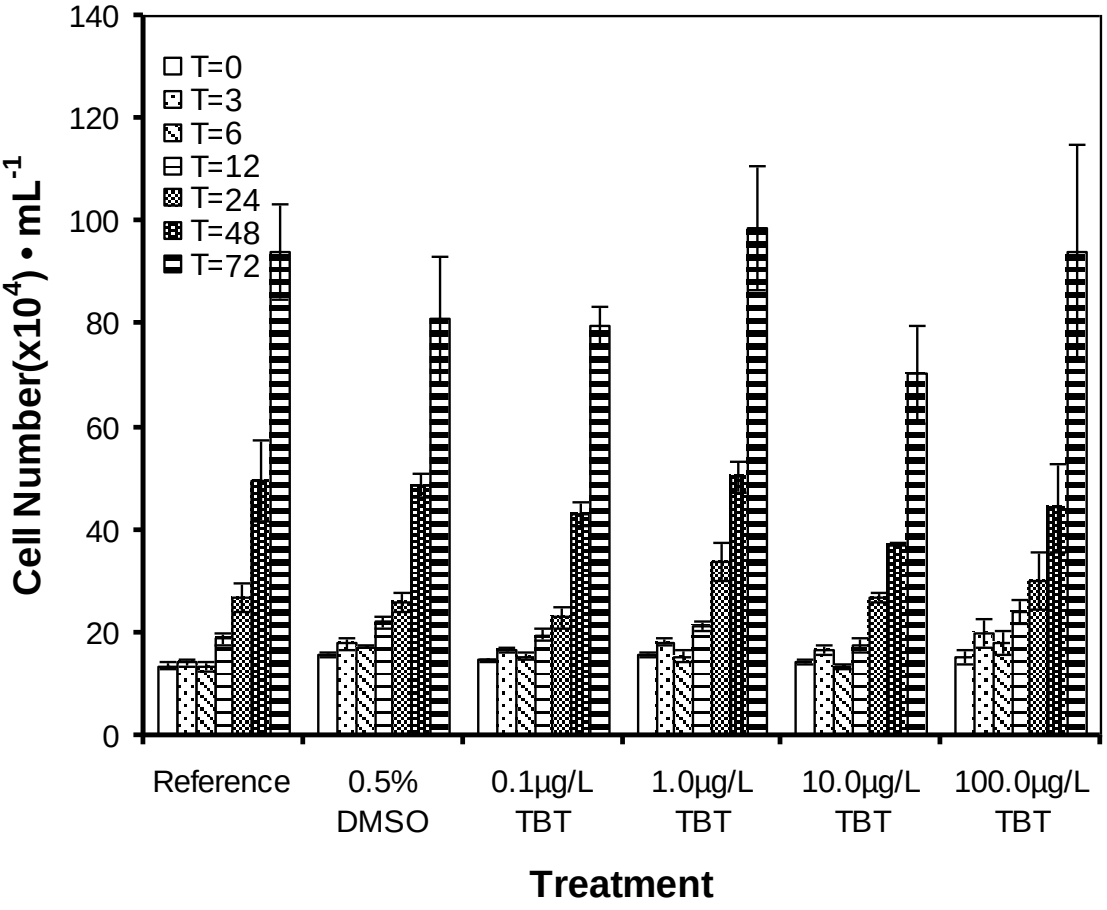


Figure 4.6: Cell counts (±SD) for *Euglena gracilis* over a 72-h (T=time (h)) exposure period in four different concentrations of TBT.

Values less than or equal to 0.05 indicated that there was a 95% probability that they were different. The lowest value presented in Table 4.10 was 0.920 and therefore indicated that all growth rates for this test were similar. It was determined that using a regression curve to perform cell counts for *Euglena* was not an appropriate test for the purpose of early detection of detrimental levels TBT in aquatic environments and this experiment was not recommended in the final multispecies early-warning biomonitoring system.

Table 4.10: Statistical analysis of cell counts of *Euglena gracilis* after 72-h comparing the reference to all other treatments. Data from Figure 12 was log-transformed and analysed by ANOVA with a Tukey *post hoc* comparison.

P-value					
Overall	0.5% DMSO	0.1µg/L TBT	1.0µg/L TBT	10.0µg/L TBT	100.0µg/L TBT
0.073	0.951	1.000	0.999	0.920	1.000

D) Behavioural Experiment #1: Light Exposure

The last type of experiments with *Euglena gracilis* explored the behavioural changes with exposure to potentially stressed situations. These tests were conducted again in both the light and in the dark. The light test was carried out for 6-h and 2 different morphologies of cells, spindle (normal) and cyst (stressed), were counted (n=3) at each sampling time (Appendix J). Normal spindle-shaped *Euglena* were selected based on a fully-elongated cell type and stressed cells were counted if they were fully contracted in their spherical cyst conformation or if they were partially contracted. Once all cell types were counted, the percentage of cyst cells was calculated and was presented in Figure 4.7. Observations of cyst-shaped *Euglena* cells increased over time for all treatments (reference, DMSO, 10 and 100 µg/L) but a relatively higher percentage was found in the 10.0 and 100.0 µg/L treatments of TBT. The carrier in the experiment showed similar cell shape trends in comparison to the reference and therefore did not appear to have an effect on the morphology of this organism. *Euglena* morphology was a good measure of stressed conditions, in the short term, when experiments were conducted in the light.

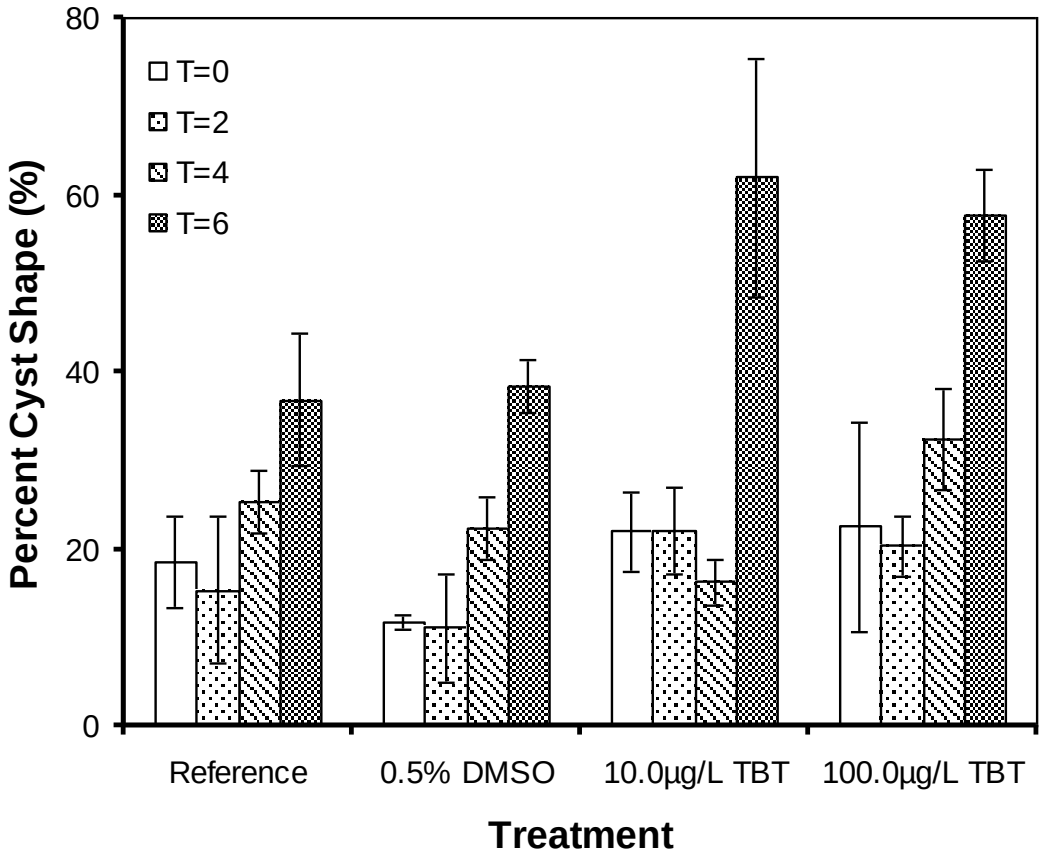


Figure 4.7: Behavioural changes, percent cyst shape (stressed) ±SD, for *Euglena gracilis* observed over a 6-h (T=time (h)) period in the light with exposure to 2 different concentrations of TBT (n=3).

E) Behavioural Experiment #2: Dark Exposure

Behavioural analysis experiments in the dark were performed in parallel with light exposure experiments. Percentage of cyst cells observed for dark experiments at indicated time increments (±SD) are presented in Figure 4.8 (Appendix K). Reference and DMSO treatments showed similar trends of approximately 60% cyst shaped cells at the end of the 6-h experiment while 10.0 and 100.0 µg/L TBT treatments present much lower percentages. At the end of the dark experiment with no exposure to TBT *Euglena* preferred to be in the cyst conformation which is usually associated with stress. This particular protist contains chloroplasts and has the ability to photosynthesis. In the short term, exposing the organism in to complete darkness acts an additional stressor which could explain the unexpected results displayed in Figure 4.8. A combination stress effect could be occurring and therefore chemical exposure experiments monitoring the morphology of *Euglena* are not recommended in the early-warning system.

4.1.4 *Anodonta grandis*

Two different parameters were observed during the freshwater mussel exposure to different concentrations of TBT. The first one was the open and closed behaviour of the mussel valves (Appendix L). The open configuration denoted that the mussel was respiring and if the valves appeared closed then it was assumed that respiration was not occurring. Results from these experiments were inconclusive. The percentages of open valves are presented in Table 4.11. The initial intent for monitoring these two parameters was to correlate the open/closed confirmation with the respiration rate (DO content).

Table14.11: Behavioural observations of mussel valve movement with exposure to different concentrations of TBT. Percentage (%) of valves open at a particular time interval (h) averaged over replicates (n=5).

	Percent of Valves Open (%)				
Time (h)	Reference	0.5%DMSO	0.1ug/L TBT	1.0ug/L TBT	10.0ug/L TBT
0.0	100	80	20	80	100
1.0	60	80	60	80	80
2.0	80	80	40	80	100
3.0	80	60	60	40	60
4.0	60	0	40	40	80
5.0	80	40	40	40	60
6.0	60	0	60	40	80

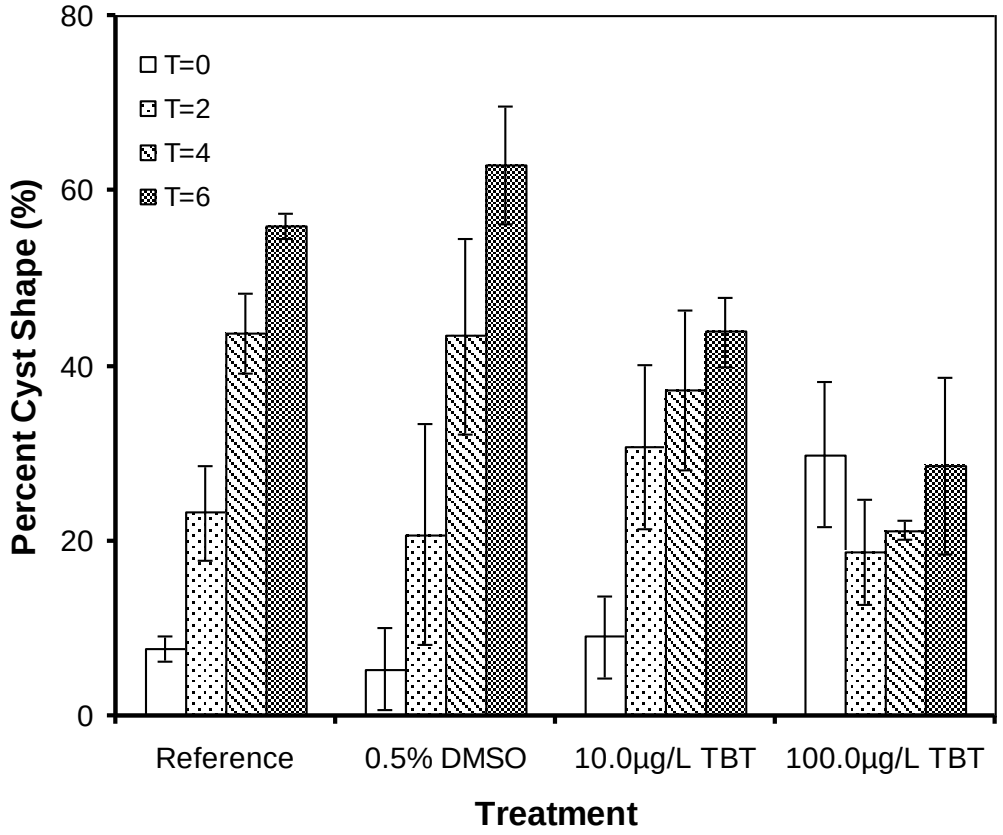


Figure 4.8: Behavioural changes, percent cyst shape (stressed) ±SD, for *Euglena gracilis* observed over a 6-h (T=time (h)) period in the dark with exposure to 2 different concentrations of TBT (n=3).

The second endpoint was to quantify the respiration rate by determining the DO content over time for each test vessel using Winkler titrations. Figure 4.9 displays the results (±SD) for the 6-h experiment with exposure to increasing concentrations of TBT. Data presented in Figure 4.9 was derived from the average respiration rates from five replicates for a given treatment (Appendix M). The reference displays the expected respiration rate per mass of the mussel under normal conditions where no influence from external toxic chemicals is exhibited. The TBT carrier, 0.5% v/v DMSO, was introduced to all the test vessels except for the reference. Vessels with only the addition of DMSO showed a similar respiration trend relative to the reference treatment. The lowest concentration of TBT, 0.1µg/L demonstrated an increased oxygen consumption rate. Greater test concentrations, 1.0 and 10.0 µg/L of TBT there was a noticeable decrease in the respiration rate in comparison to the 1.0 µg/L treatment. ANOVA tests with a Tukey *post hoc* comparison were conducted on the respiration rates and p-values determining if treatments were significantly different from the reference are displayed in Table 4.12.

Values less than or equal to 0.05 would show a significant difference from the reference. This statistical analysis did not show that there was any difference amongst any of the treatments when compared to the reference. Freshwater mussels are very sensitive organisms and respiration was a good indicator of stress. There was a noticeable increasing respiration trend with the addition of the lower concentration of TBT. Therefore, bivalves would contribute a fast response indication of undesirable surrounds and recommended for further testing.

Table 4.12: Probability that TBT treatments respiration rates for *Anodonta grandis* are significantly different from the reference treatment.

P-value				
Overall	0.5% DMSO	0.1 µg/L TBT	1.0 µg/L TBT	10.0 µg/L TBT
0.042	0.997	0.253	0.998	0.733

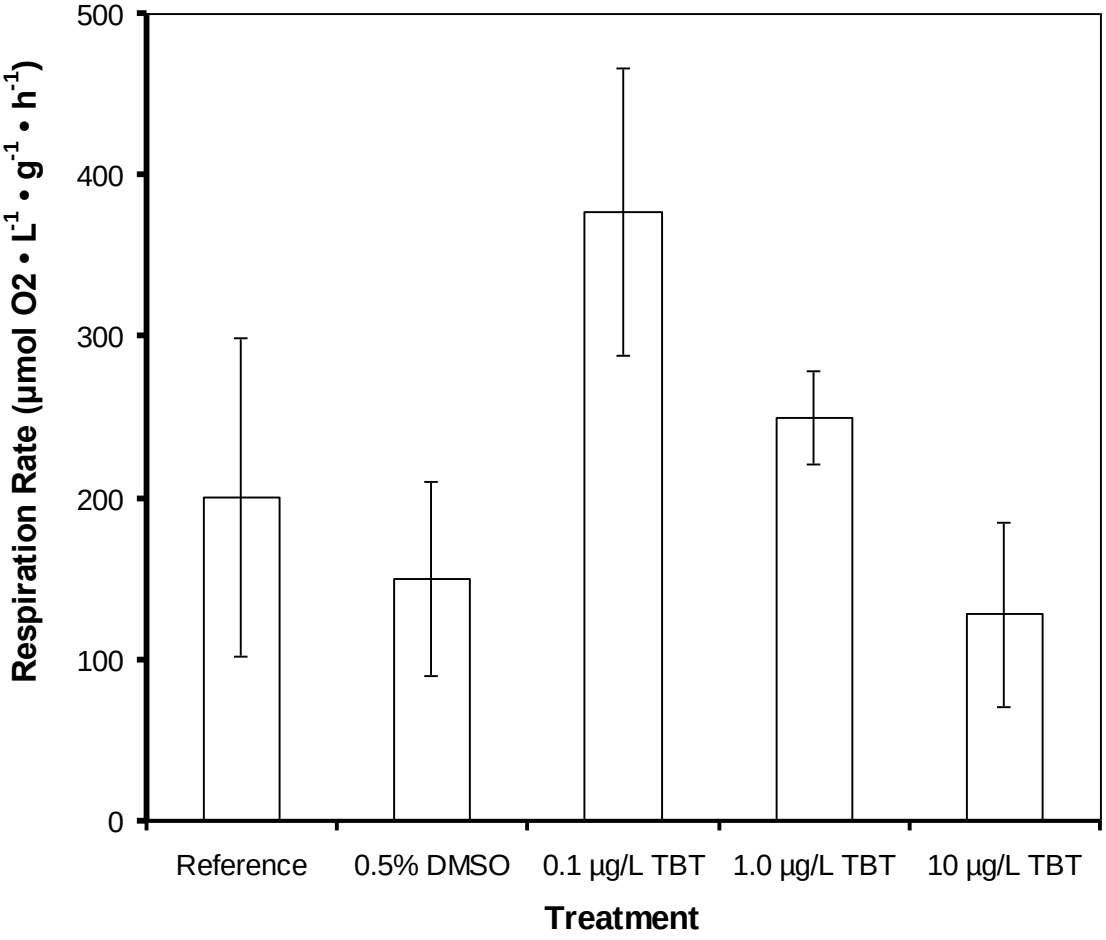


Figure 4.9: Average respiration rate (µmol $O_2 \cdot L^{-1} \cdot g^{-1} \cdot h^{-1}$) of *Anodonta grandis* (±SD) for a given treatment of TBT (n=5).

4.2 Bioassay organism responses to atrazine

Identical toxicity bioassays were performed with the second chemical of interest in this research, atrazine, following the same protocols used for experimentation with TBT. All indicator organisms were subject to the same test with alterations in chemical concentrations due to the environmental relevance and allowable limits in aquatic waters.

4.2.1 *Lemna minor*

Changes to the organism's growth rate were monitored over a 96-h test period (Appendix N) by making frond counts in 24-h increments. Figure 4.10 outlines the results obtained from the growth inhibition experiments relating frond counts to the growth rate of the macrophyte. The reference treatment represents a normal growth progression of the plant species and is compared to the addition of the carrier, DMSO and also three concentrations of atrazine. Exposure to 0.5% DMSO showed to have a similar growth pattern in relation to the reference treatment. With the addition of 5.0 and 50.0 µg/L of atrazine, the ability of the plant to grow new fronds is slightly retarded. After a 24-h exposure to 500.0 µg/L of atrazine, it was observed that these macrophytes are unable to reproduce and the growth rate plateaus for the remainder of the 96-h experiment. Statistical analysis was further conducted on the growth rate date to determine if there was a significant difference in comparison with the reference.

Differences between the reference growth rate and all other treatments were analysed using ANOVA with a Tukey pair-wise *post hoc* comparison to determine the probability that there is a difference between slopes (growth rates). Table 4.13 outlines the p-values comparing the reference to each treatment. Values less than 0.05 showed there is significant difference between the growth rates. This represented a 95% probability that these growth rates were different in comparison to the reference test. Significant difference from the reference was only shown with exposure to 500.0 µg/L of atrazine. *L. minor* is sensitive to relatively higher concentrations of atrazine, 500 µg/L, and would detect contaminated waters at the sub-acute level. Therefore, this macrophyte should be considered in the development of an early-warning biomonitoring system.

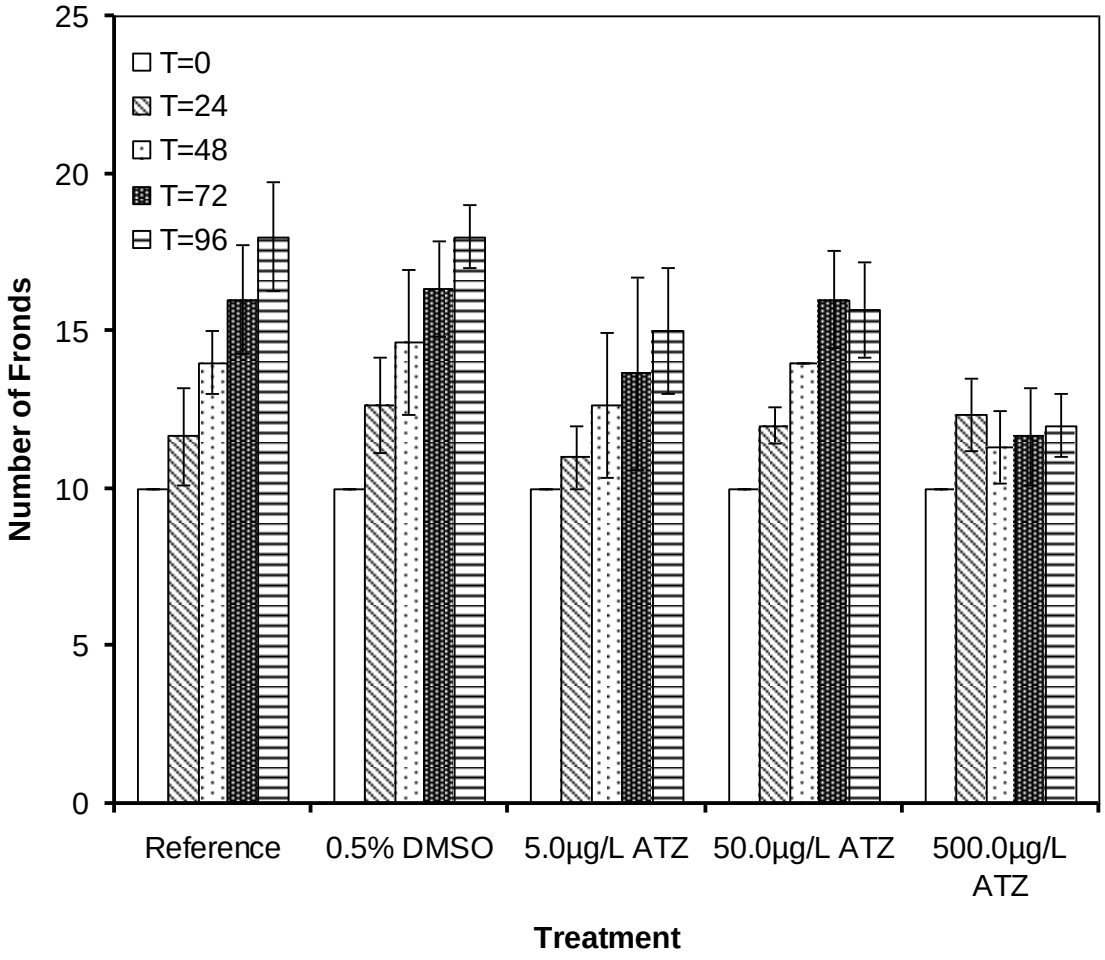


Figure 4.10: Frond counts for *Lemna minor* ±SD over a 96-h (T=time (h)) exposure period to three different concentrations of atrazine.

Table 4.13: ANOVA of *Lemna minor* growth rate over 96-h comparing the reference to all other treatments of atrazine.

P-value				
Overall	0.5% DMSO	5.0µg/L Atrazine	50.0µg/L Atrazine	500.0µg/L Atrazine
0.001*	0.970	0.301	0.216	0.001*

*Statistically significant values

4.2.2 *Pseudokirchneriella subcapitata*

Three tests were conducted with *Pseudokirchneriella subcapitata* with exposure to selected concentrations of atrazine. Two of the tests analysed the biochemical endpoints, photosynthesis and respiration, and then the third test investigated the cell proliferation over 72-h.

A) Biochemical Endpoint #1: Photosynthetic Rate (Light experiments)

The average DO content in light exposure experiments for individual sampling periods and the photosynthetic rate (slope) are outlined in Table 4.14 (Appendix O). DO content was quantified using Winkler titrations for experiments done in triplicate (n=3). The slopes for individual treatments in Table 4.14 are representative of the columns in Figure 4.11. Declining O_2 concentrations from respiration delivers a negative slope and therefore the slope was multiplied by negative one to give positive values. The photosynthetic rates in Figure 4.11 display how the algae were affected by two concentrations of atrazine. 0.5% v/v DMSO had a similar photosynthetic rate to the reference. There was a noticeable decreasing trend in oxygen production with a 6-h exposure to 50.0 µg/L of atrazine and a depletion of oxygen concentrations with exposure to the 500.0 µg/L of atrazine. The data from light experiments were statistically analysed and presented in Table 4.15. Individual vessels were analysed by determining the equality of two correlations using the Kruskal-Wallis test which compared the reference photosynthetic rates with all other treatments. These statistical tests produced p-values which indicated if there were rate correlations and are displayed in Table 4.15 for light experiments. A p-value of less than or equal to 0.05 indicates a significant difference in photosynthetic rate with a 95% probability that the rates are different. This experiment showed that there was a significant difference amongst the reference and the highest concentration of atrazine.

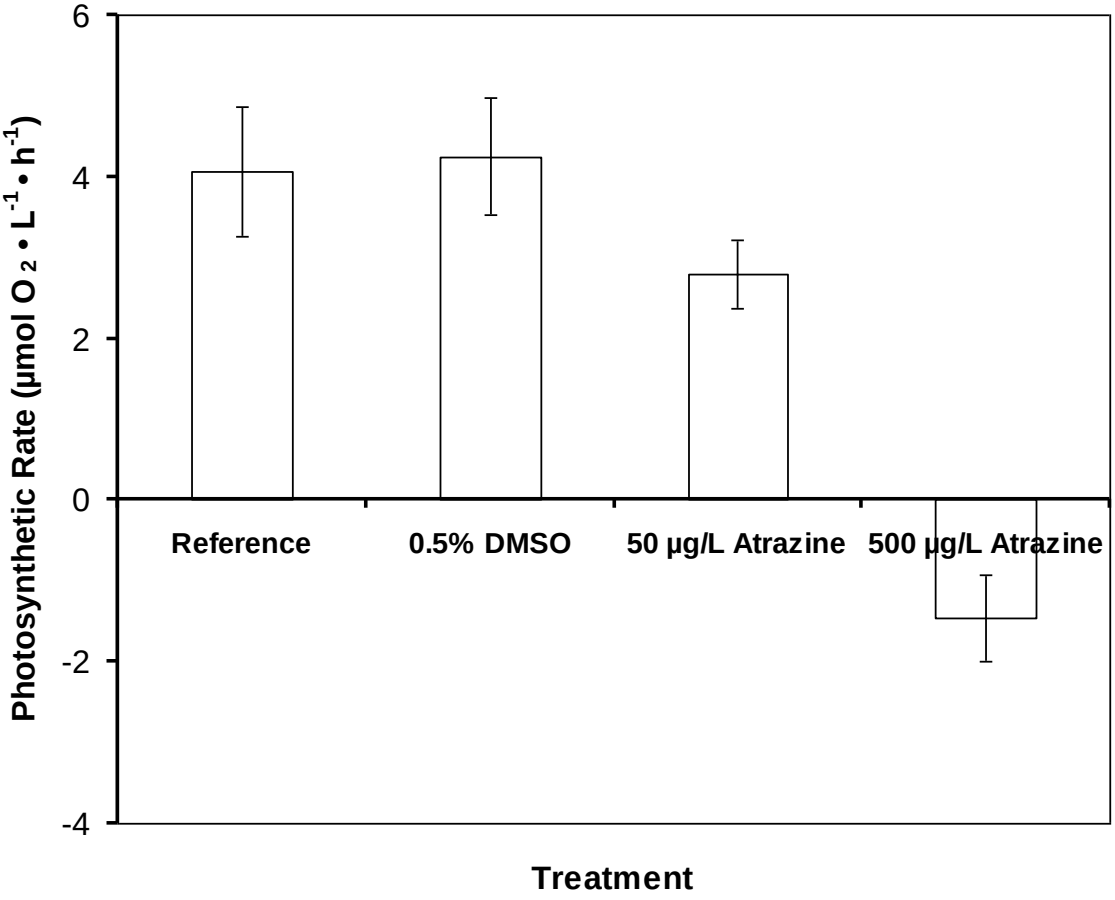


Figure 4.11: Photosynthetic rate (µmol $O_2 \cdot L^{-1} \cdot h^{-1}$) of *Pseudokirchneriella subcapitata* (±SD) exposed to two concentrations of atrazine derived from 6-h light exposure experiments (n=3). Rates were determined from data presented in Table 4.14.

Table 4.14: DO content (µmol $O_2 \cdot L^{-1}$) for light experiments with exposure to two concentrations of atrazine. Replicates (n=3) were averaged in corresponding sampling periods (±SD). The slope (µmol $O_2 \cdot L^{-1} \cdot h^{-1}$) represents the photosynthetic rate of the algae over the 6-h experiment.

	Average DO Content (µmol O_2/ L) per Treatment (n=3)			
Time (h)	Reference (± SD)	0.5% v/v DMSO (± SD)	50 µg/L Atrazine (± SD)	500 µg/L Atrazine (±SD)
0	0.2229 (0.0032)	0.2208 (0.0050)	0.2223 (0.0032)	0.2167 (0.0039)
1	0.2347 (0.0063)	0.2337 (0.0009)	0.2260 (0.0039)	0.2192 (0.0063)
2	0.2415 (0.0032)	0.2389 (0.0016)	0.2327 (0.0016)	0.2172 (0.0056)
3	0.2430 (0.0054)	0.2441 (0.0024)	0.2327 (0.0027)	0.2172 (0.0031)
4	0.2347 (0.0078)	0.2373 (0.0041)	0.2301 (0.0018)	0.2110 (0.0068)
5	0.2430 (0.0050)	0.2389 (0.0027)	0.2353 (0.0032)	0.2104 (0.0047)
6	0.2575 (0.0047)	0.2575 (0.0047)	0.2430 (0.0047)	0.2110 (0.0056)
Slope	**0.0041**	**0.0042**	**0.0028**	**-0.0015**

With exposure to the highest concentration of atrazine (500 µg/L), it showed to have a negative production of O_2 indicating significant production in CO_2 due to cellular respiration. This accounts for the negative rate in Figure 4.11. Therefore this endpoint is considered to be an early-warning parameter for this species of green algae when exposed to atrazine.

Table 4.15: Statistical investigation for *Pseudokirchneriella subcapitata* photosynthesis experiments of the equality of two correlations comparing the reference with each treatment of atrazine.

P-value		
0.5% DMSO	50.0 µg/L Atrazine	500.0 µg/L Atrazine
0.722	0.530	<0.001*

*Statistical significance

B) Biochemical Endpoint #2: Respiration Rate (Dark experiments)

Biochemical analysis of oxygen content was also performed in the dark. Results from dark exposure tests, including average DO content and photosynthetic rate (slope), are outlined in Table 4.16 (Appendix P). The slopes from Table 4.16 are also presented in Figure 4.12 with standard deviations. DO content was quantified using Winkler titrations for experiments done in triplicate (n=3).

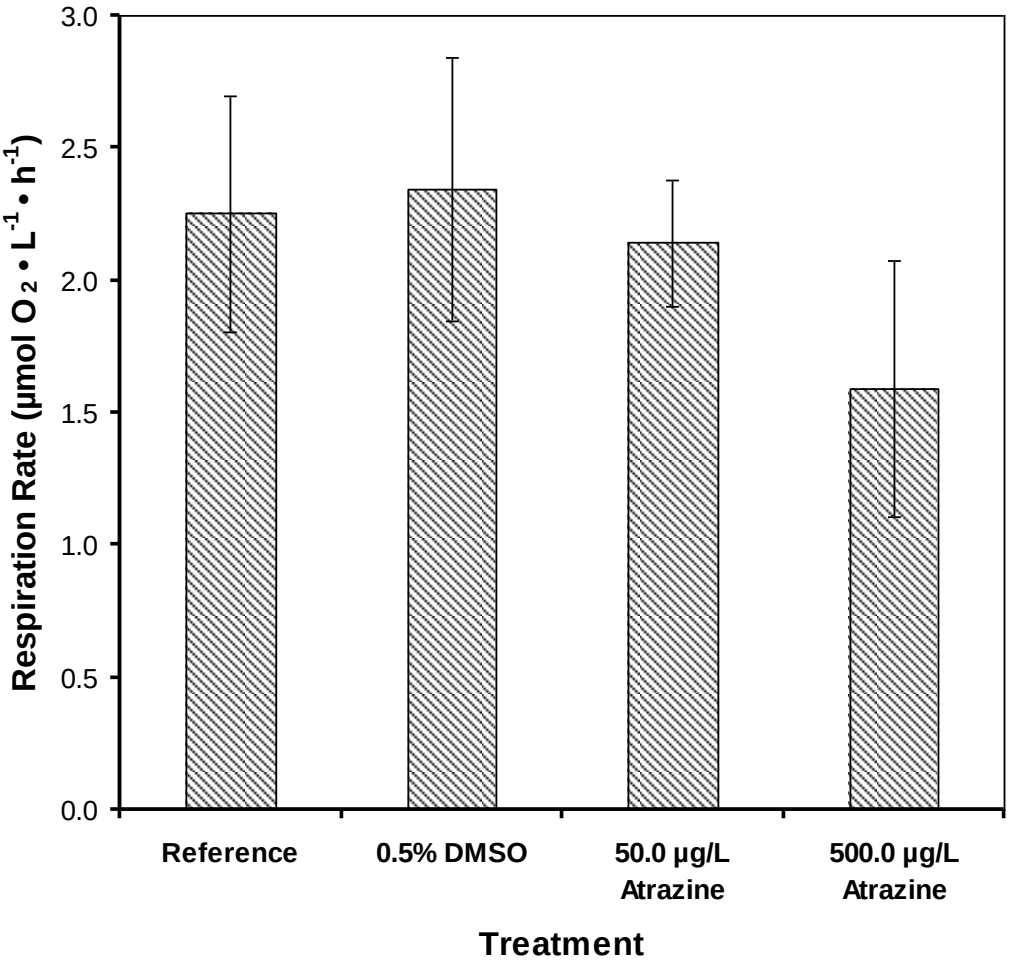


Figure24.12: Respiration rate (µmol $O_2 \cdot L^{-1} \cdot h^{-1}$) of *Pseudokirchneriella subcapitata* (±SD) exposed to two concentrations of atrazine derived from 6-h dark exposure experiments. Rates were determined from data presented in Table 4.16.

Table 4.16: DO content (µmol $O_2 \cdot L^{-1}$) for dark experiments with exposure to two concentrations of atrazine. Replicates (n=3) were averaged in corresponding sampling periods (±SD). The slope (µmol $O_2 \cdot L^{-1} \cdot h^{-1}$) represents the respirations rate of the algae over the 6-h experiment.

	Average DO Content (µmol $O_2 \cdot L^{-1}$) per Treatment (n=3)			
Time (h)	Reference (± SD)	0.5% v/v DMSO (± SD)	50 µg/L Atrazine (± SD)	500 µg/L Atrazine (± SD)
0	0.2198 (0.0024)	0.2182 (0.0065)	0.2182 (0.0009)	0.2182 (0.0039)
1	0.2151 (0.0063)	0.2182 (0.0054)	0.2172 (0.0016)	0.2130 (0.0024)
2	0.2161 (0.0024)	0.2192 (0.0024)	0.2172 (0.0016)	0.2187 (0.0022)
3	0.2115 (0.0045)	0.2141 (0.0047)	0.2141 (0.0016)	0.2130 (0.0032)
4	0.2058 (0.0047)	0.2058 (0.0036)	0.2089 (0.0024)	0.2048 (0.0041)
5	0.2073 (0.0039)	0.2068 (0.0018)	0.2068 (0.0024)	0.2079 (0.0000)
6	0.2073 (0.0032)	0.2084 (0.0024)	0.2079 (0.0016)	0.2115 (0.0032)
Slope	**0.0023**	**0.0023**	**0.0021**	**0.0016**

Respiration rates for all treatments in dark experiments exposed to atrazine are shown in Figure 4.12. The reference, DMSO and 50.0 µg/L of atrazine treatments, displayed similar photosynthetic trends where the 500.0 µg/L had a noticeable decline in oxygen depletion. Statistical analysis using the Kruskal-Wallis test was used to determine if there was a correlation between the respiration rates of the reference and the other treatments. This statistical test produced p-values (Table 4.17) which indicated if there was a correlation amongst the reference and test treatments. None of the p-values for this test was less than or equal to 0.05, which is necessary for the significance of the data. Monitoring the respiration rate (dark exposure) of the green algae did not produce significant results and was not an ideal parameter for green algae to detect a contaminated environment at the sub-acute level.

Table 4.17: Statistical analysis for *Pseudokirchneriella subcapitata* respiration experiments of the equality of two correlations comparing the reference with each treatment of atrazine.

P-value		
0.5% v/v DMSO	50.0 µg/L Atrazine	500.0 µg/L Atrazine
0.877	0.148	0.379

C) Growth inhibition test (Cell counts)

Cell counts were observed and complied over a 72-h period for each individual treatment. Figure 4.13 shows the growth for all treatments over the experimental period (Appendix Q). Cell counts were developed using a regression curve (Figure 3.4) which

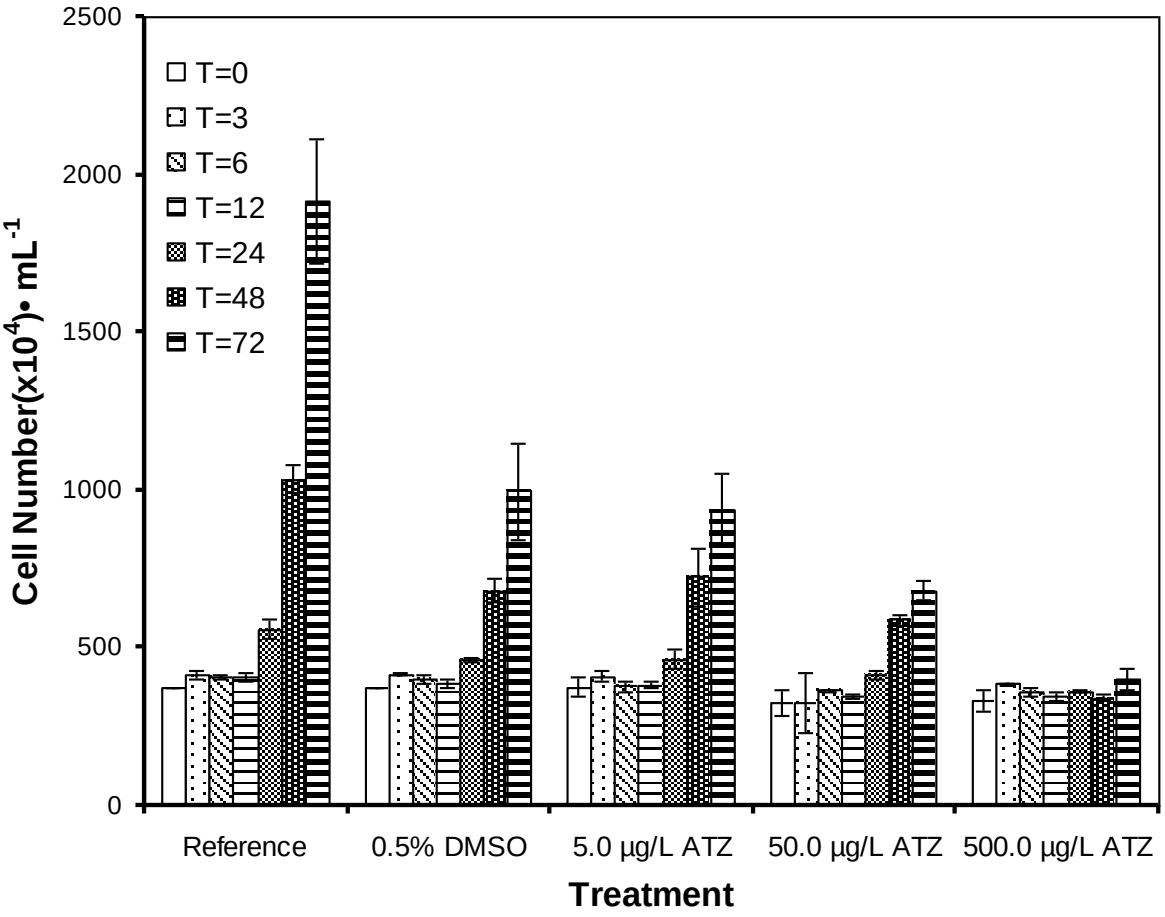


Figure 4.13: Cell counts (±SD) for *Pseudokirchneriella subcapitata* over a 72-h (T=time (h)) exposure period to three different concentrations of atrazine.

accurately compared cell numbers to OD at 650 nm. Exposure to 0.5% v/v DMSO had an effect on the growth rate *Pseudokirchneriella subcapitata* which can be visually interpreted in Figure 4.13 and statistically in Table 4.18. Table 4.19 was constructed to remove the compound effects of the carrier and target the effects of the test chemical. Exposure to 5.0 and 50.0 µg/L of atrazine did not appear to have a toxic effect trend. The growth rate of the algae quickly plateaued after a 24-h exposure to 500.0 µg/L of atrazine. Statistical analysis for these growth inhibition experiments were conducted using ANOVA with a Tukey pair-wise *post hoc* comparison. Table 4.18 uses the reference as an expected growth rate for comparison. Due to the effects of the carrier for this experiment, Table 4.19 was produced to use the DMSO treatment as the reference

treatment to eliminate toxicity effects influenced by the carrier chemical. P-values less than or equal to 0.05 indicated there was a 95% probability that there was a difference in growth rates form the reference treatment. In Table 4.19, only the 500.0 µg/L of atrazine produced a p-value of less than 0.05. This test proved that this parameter has potential in delivering sub-acute response when exposed to concentrations equal to or higher than 500 µg/L of atrazine and therefore should be considered for the development of an early-warning biomonitoring system.

Table 4.18: Statistical analysis of the growth rate of *Pseudokirchneriella subcapitata* over a 72-h period comparing the reference to all other treatments of atrazine. Data from Figure 25 was log transformed and analysed by ANOVA with a Tukey *post hoc* comparison.

P-value				
Overall	0.5% DMSO	5.0µg/L Atrazine	50.0µg/L Atrazine	500.0µg/L Atrazine
<0.001*	0.001*	0.001*	<0.001*	<0.001*

*Statistically significant values

Table 4.19: Statistical analysis of the growth rate of *Pseudokirchneriella subcapitata* over a 72-h period comparing the 0.5% v/v DMSO to all other treatments of atrazine. ANOVA was conducted on log transformed growth rates. Data from Figure 25 was log transformed and analysed by ANOVA with a Tukey *post hoc* comparison.

P-value			
Overall	5.0µg/L Atrazine	50.0µg/L Atrazine	500.0µg/L Atrazine
<0.001*	1.000	0.504	<0.001*

*Statistically significant values

After all three different experiments with the green algae, it was determined that *P. subcapitata* responded well as an early-warning bioindicator. Two of the three experiments, photosynthetic rate and cell inhibition tests, are recommended for further experimentation with alternate chemicals to determine the sensitivity at the sub-acute level.

4.2.3 *Euglena gracilis*

Euglena underwent three types of toxicity bioassays: biochemical (monitoring oxygen concentrations), growth rate (cell counts), and behavioural analysis (cell morphology). Each test investigated different parameters to see which would deliver the quickest results for this indicator organism.

A) Biochemical Endpoint #1: Respiration Rate (Light experiments)

Euglena was monitored for changes in oxygen development in the light with exposure to two different concentrations of atrazine (Appendix R). Table 4.20 displays that there was a non-linear relationship of the data which is denoted by the low r^2 values. Further investigation of this data was not conducted due to the drastic fluctuations in oxygen concentrations at each sampling time. Similar results occurred as in the TBT experiments, monitoring the respiration rate of *Euglena* did not show that the organism was experiencing stressful conditions Therefore, this experiment was not recommended for the development of an early-warning detection system.

Table 4.20: Respiration rate (µmol $O_2 \cdot L^{-1} \cdot h^{-1}$) of *Euglena gracilis* exposed to two concentrations of atrazine derived from 6-h light exposure experiments.

Treatment	Slope (µmol $O_2 \cdot L^{-1} \cdot h^{-1}$)	r^2 value
Reference	-1.358	0.1491
0.5% DMSO	-1.1265	0.0456
50.0µg/L Atrazine	-0.1847	0.002
500.0µg/L Atrazine	0.1293	0.0043

B) Biochemical Endpoint #2: Respiration Rate (dark experiments)

Biochemical experiments were also conducted in the dark to isolate the respiratory functions of *Euglena* and eliminate photosynthetic capabilities (Appendix S). Figure 4.14 displays the respiration rates (±SD) of *Euglena gracilis* when tests were conducted in the dark. In this figure it can be observed that there is no difference between the respiration rates for all treatments. Statistical analysis (Table 4.21) was conducted to determine if there was a difference in the respiration rates for this bioassay. Respiration rates were analysed using the Kruskal-Wallis test to statistically determine if there was

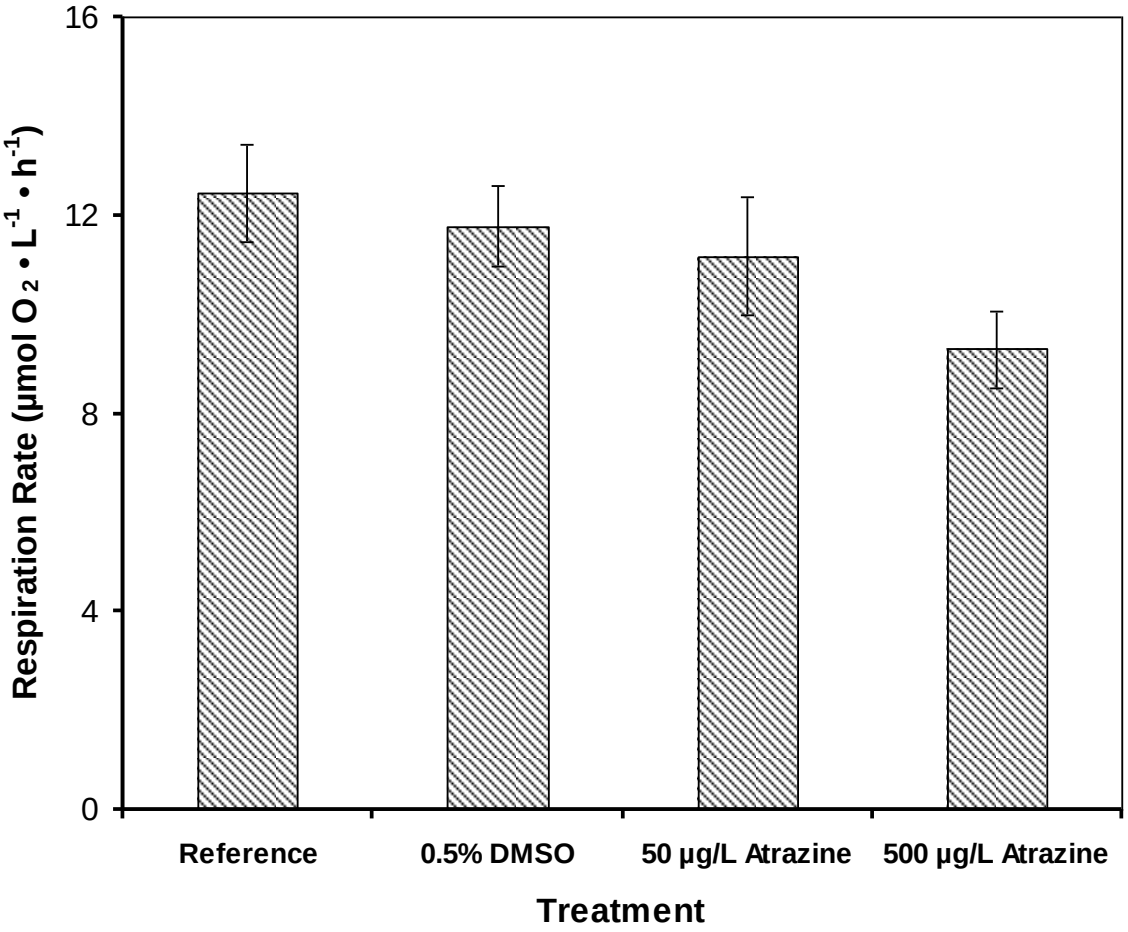


Figure 4.14: Respiration rate (µmol $O_2 \cdot L^{-1} \cdot h^{-1}$) of *Euglena gracilis* (±SD) exposed to two concentrations of TBT derived from 6-h dark exposure experiments.

equality of the correlations when comparing the reference to all other treatments. P-values less than 0.05 indicate a 95% probability that the treatment respiration rates are different from the reference. Table 4.21 illustrates that there was no significant difference among all treatments. Dark experiments with exposure to atrazine show that, monitoring the respiration rate of *Euglena* was not a useful measurement for early detection of any experimental atrazine concentrations used in this study. Therefore was not recommended for further testing with this specific organism and chemical.

Table24.21: Statistical investigation for *Euglena gracilis* respiration experiments of the equality of two correlations comparing the reference with each treatment of atrazine.

P-value		
0.5% DMSO	50.0µg/L Atrazine	500.0µg/L Atrazine
0.736	0.374	0.823

*Statistical significance

C) Growth inhibition test (Cell counts)

Cell count tests were conducted over a 72-h period (Appendix T) using absorbance readings and a regression curve (Figure 3.6). Figure 4.15 shows all cell counts over the duration of the experiment for all treatments. Statistical analysis of the cell count data is presented in Table 4.22. Data in Figure 4.15 produced a growth rate and this slope was log transformed prior to ANOVA tests with a Tukey *post hoc* comparison. The log transformation allowed for linear regression analysis to determine if atrazine exposure affected the growth rates of the protist. Figure 4.15 delivers appropriate observations that there is no difference in the growth rates for any treatments with atrazine. Statistical analysis of the cell counts using ANOVA was performed to determine if the atrazine treatments had a significant correlation with the reference and is presented in Table 4.22. P-values less than or equal to 0.05 would indicate that there was a 95% probability that they were different. The lowest value in Table 4.22 was 0.962 and therefore indicated that all growth rates for this test were similar. The *Euglena* growth inhibition test did not produce significant results and was not recommended for use to identify atrazine in the final multispecies early-warning biomonitoring system.

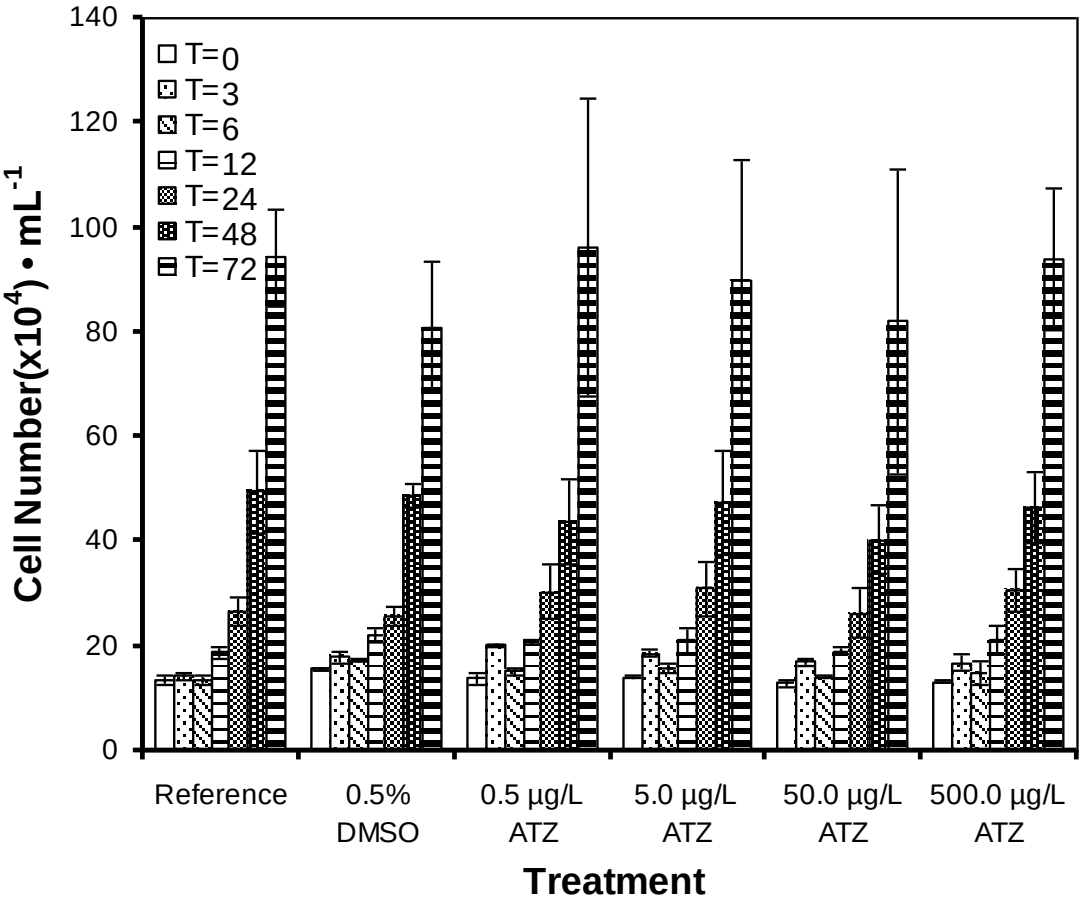


Figure 4.15: Cell counts for *Euglena gracilis* (±SD) over a 72-h (T=time (h)) exposure period to four different concentrations of atrazine.

Table 4.22: Statistical analysis of cell counts of *Euglena gracilis* at 72-h period comparing the reference to all other treatments. Data from Figure 27 was log transformed and analysed by ANOVA with a Tukey *post hoc* comparison.

			P-value		
Overall	0.5% DMSO	0.1µg/L Atrazine	1.0µg/L Atrazine	10.0µg/L Atrazine	100.0µg/L Atrazine
0.906	0.962	1.000	1.000	0.972	1.000

D) Behavioural Experiment #1: Light Exposure

Behavioural tests for *Euglena gracilis* were conducted over a 6-h period in the light and cell counts of two different morphologies, spindle (normal) and cyst (stressed) (Figure 3.5), were counted (Appendix U). Normal shaped (spindle) *Euglena* were selected based on a fully elongated cell types and stressed cells (cyst) were counted if they were fully contracted in their spherical cyst conformation or if they were partially contracted. Once all cell types were counted the percentage of cyst cells was calculated and was presented in Figure 4.16. The carrier, DMSO, showed similar cell shape trends to the reference and therefore did not appear to have an effect on the morphology of this organism. Stressed cells percentages increased over the course of the experiment for all treatments but 50.0 and 500.0 µg/L treatments of atrazine contained a higher percentage of stressed cells (cyst) by the end of the test period. Experiments conducted on *Euglena* with exposure to atrazine in the light produced significant data which indicated that the test chemical caused a change in the morphology of the organism. Therefore, cell-shape under light conditions was recommended for the development of the multispecies system.

E) Behavioural Experiment #2: Dark Exposure

Behavioural analyses in the dark were performed in parallel with light exposure experiments. Percentage of cyst cells observed for dark experiments at indicated time increments (±SD) are presented in Figure 4.17 (Appendix V). All treatments showed similar trends of approximately 60% cyst shaped cells by the end of the 6-h experiment. At the end of the dark experiment with no exposure to atrazine *Euglena* preferred to be in the cyst conformation which is usually associated with stress. The chloroplasts contained within the body of the protist, allow the organism to undergo photosynthetic reactions.

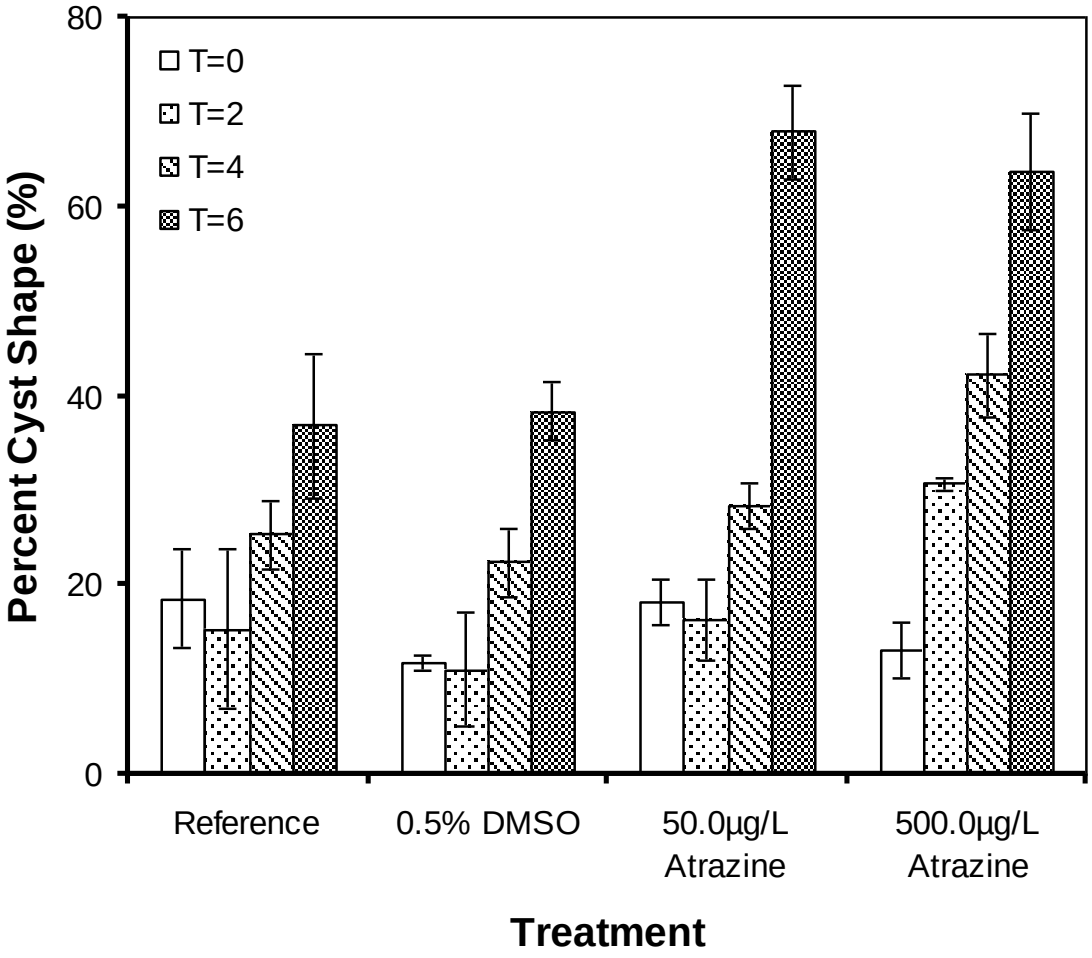


Figure 4.16: Behavioural changes, percent cyst shape (stressed) ±SD, for *Euglena gracilis* observed over a 6-h (T=time (h)) period in the light with exposure to two different concentrations of atrazine (n=3).

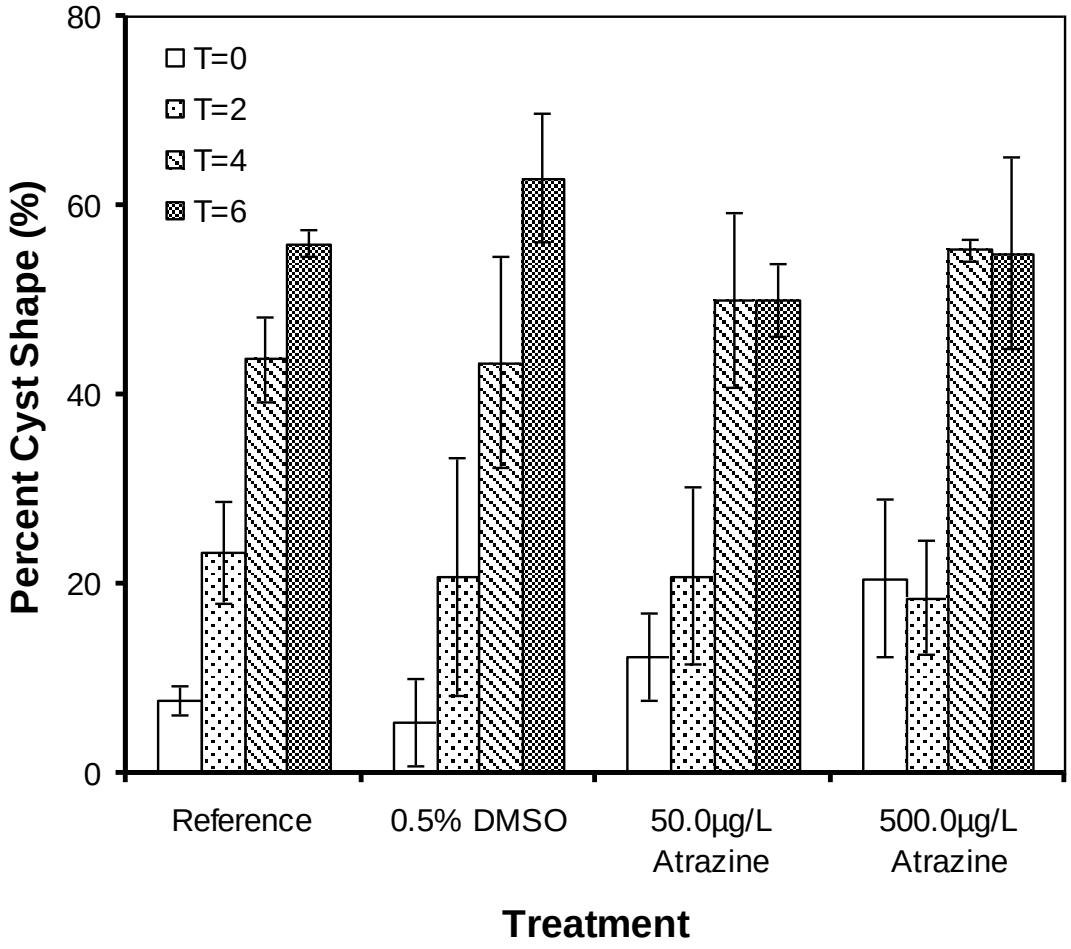


Figure 4.17: Behavioural changes, percent cyst shape (stressed) ±SD, for *Euglena gracilis* observed over a 6-h (T=time (h)) period in the dark with exposure to two different concentrations of atrazine (n=3).

Abruptly exposing the organism to complete darkness poses stressful conditions and explains the unexpected results displayed in Figure 4.17. Experiments monitoring the morphology of *Euglena* conducted in dark conditions were not recommended for the early-warning system.

4.2.4 *Anodonta grandis*

Quantifying the respiration rate by determining the DO content over time for each test vessel was accomplished via Winkler titrations. Figure 4.18 displays the results (±SD) for a 6-h experiment with exposure to three concentrations of atrazine, 5.0, 50.0 and 500.0 µg/L. Data presented in Figure 4.18 was derived from the average respiration rates from 5 replicates for a given treatment (Appendix W). The reference displays the expected respiration rate per mass of the mussel under normal conditions where no influence from external toxic chemicals is exhibited. Mussel exposure to 0.5% v/v DMSO had a similar respiration rate compared to the reference. Increased concentrations of atrazine displayed a decreasing trend in the respiration rate of the mussels. 500.0 µg/L of atrazine caused the average respiration of the mussels to decrease to 50 µmol $O_2 \cdot L^{-1} \cdot g^{-1} \cdot h^{-1}$.

The respiration rates (slope) were analysed using an ANOVA test to determine if there was a significance change in respiration rate. ANOVA with a Tukey *post hoc* comparison were conducted on the respirations rates and p-values determining if treatments were significantly different from the reference are displayed in Table 4.23. P-values less than or equal to 0.05 showed a significant difference from the reference. This statistical analysis showed that the 5.0 and 500.0 µg/L treatments had a 95% probability that the respiration rates were different from the reference. *A. grandis* demonstrated that it is a sensitive organism with respect to its respiration rate. The respiration rate of this organism displayed sub-acute responses with exposure to atrazine, identifying unfavourable aquatic conditions. Therefore, it was recommended for further testing with other chemicals in the development of a multispecies early-warning biomonitoring system.

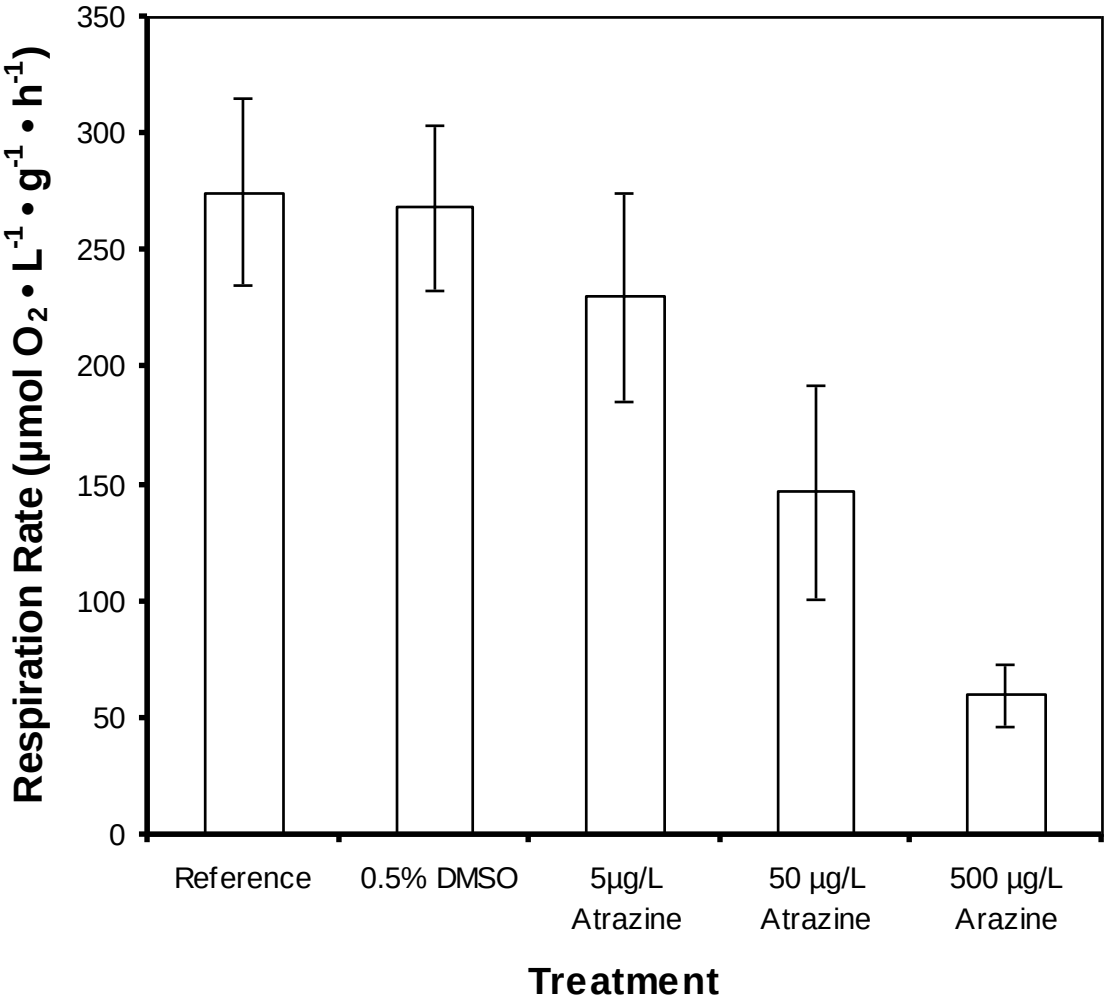


Figure 4.18: Average respiration rate (µmol $O_2 \cdot L^{-1} \cdot g^{-1} \cdot h^{-1}$) of *Anodonta grandis* for a given treatment of atrazine (±SD, n=5).

Table 4.23: Probability that atrazine treatments respiration rates for *Anodonta grandis* are significantly different from the reference treatment.

P-value				
Overall	0.5% DMSO	0.5µg/L Atrazine	5.0µg/L TBT Atrazine	50.0µg/L Atrazine
<0.001*	1.000	0.661	0.007*	<0.001*

*Statistically significant

4.3 SUMMARY

Individual chemical analyses with exposure to TBT and atrazine were conducted on each organisms and an overview of the results are presented below. Figure 4.19 outlines the overall results, indicating which parameter showed to have an effect with exposure to a particular concentration of a chemical.

Lemna minor was investigated as an early-warning indicator for aquatic contaminants. Frond counts were measured and directly representative of the growth rate. Over a 96-h growth inhibition test, significant decline in growth was observed with exposure to TBT concentrations as low as 10.0 µg/L ($p<0.022$, Table 4.1). Dissolved oxygen was an alternative endpoint but due to the surface floating behaviour of the macrophyte, it would have been difficult to create a closed environment, eliminating external oxygen from affecting final results. Figure 4.1 displays that with exposure to 100.0 µg/L of TBT there was a significant decline in the growth rate after the 96-h test. Also, there was a noticeable decreasing growth trend after 24-h in comparison to the reference samples.

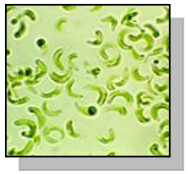
In *L. minor* tests involving atrazine (Figure 4.10) it was found that 500.0 µg/L of atrazine had significant effects ($p=0.001$, Table 4.13) on the growth rate. It is also important to mention that the 0.5% v/v DMSO treatment did not have a significant effect ($p=0.925$) and therefore, played as an ideal carrier for TBT and atrazine. A digital camera was used to take still photos of the organisms in their test vessels and manual observations of frond counts were recorded. Automation of this experiment could be

Lemna minor

Chemical	Growth Test
TBT	100 µg/L
Atrazine	500 µg/L

= No Effect

Pseudokirchneriella subcapitata

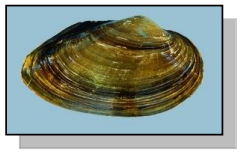


Chemical	Photosynthesis (light)	Respiration (dark)	Cell Counts
TBT	100 µg/L		100 µg/L
Atrazine	500 µg/L		500 µg/L

Euglena gracilis

Chemical	Respiration (light)	Respiration (dark)	Cell Counts	Shape (light)	Shape (dark)
TBT				10 µg/L	
Atrazine				50 µg/L	

Anodonta grandis



Chemical	Respiration	Behaviour* (open/closed)
TBT	1.0 µg/L	
Atrazine	50 µg/L	

Figure 4.19: Summary of results from all experiments conducted on *Lemna minor, Pseudokirchneriella subcapitata, Euglena gracilis* and *Anodonta grandis* outlining the effective concentrations with exposure to the test chemicals, tributyltin-hydride (TBT) and atrazine.

easily accomplished via time-lapse digital photography and a computer program which could measure surface area of the fronds. Changes in frond surface area over time would represent the growth rate for *L. minor* and real-time comparisons with reference samples could be conducted automatically.

Photosynthesis, respiration and growth rate were the three endpoints displayed for *P. subcapitata*. No concentration of TBT in the 6-h experiment was shown to have an effect on the photosynthetic or respiration rates. The results in Figure 4.4 display the 72-h growth rates of this particular organism. The exponential growth rate in Figure 4.4 was log transformed prior to linear regression analysis. The 0.5% v/v DMSO showed an effect ($p=0.006$) on the normal growth rate which was seen in Table 4.6. Table 4.7 was produced to compare the effects of only the targeted chemical, TBT, and not the carrier. There was a significant difference of growth rate in the 100.0 µg/L of TBT treatment when compared to exposure to TBT.

Photosynthesis experiments exposing the green algae (Figure 4.11) to atrazine concentrations of 500.0 µg/L had significant effects, $p<0.001$ (Table 4.15) and no selected concentrations displayed any effects in the atrazine respiration experiments (Figure 4.12). Algal growth inhibition experiments analyses with exposure to atrazine were conducted comparing the DMSO exposure to all other treatments due to the effects of the carrier in this test. Atrazine concentrations of 500.0 µg/L showed significant effects on the growth rate of the algae (Figure 4.13)

Automation of the algae growth rate experiments could be possible by constructing a flow-through system which periodically pumps a sample into a spectrophotometer that reads the OD at 650-nm and conducts a reference absorbance reading to compare both samples. Manual sampling also takes minimal time to produce results. This set up required the least amount of algae stock and growth medium in comparison to other tests conducted with algae. Once the growth inhibition experiments were set up, sampling periods only took 15 minutes to extract samples, place them in a 96-well microplate and retrieve OD readings.

Experiments conducted with *P. subcapitata* were performed on *E. gracilis* with an additional test looking at the behavioural changes of this motile protist. The biochemical experiments (DO content) conducted in the light for both TBT and atrazine resulted in non-linear plots. Relatively low r^2 values (Tables 4.8 and 4.20 for TBT and atrazine respectively) were produced for individual replicates. This non-linear fit could be due to the competing photosynthetic rate and respiratory abilities of *E. gracilis*. It is difficult to isolate and identify fluctuations in DO when a light source is present. Therefore, removal of the light source halted the photosynthetic ability of the organism and targeting specifically the respiration rate. A trend of decreased respiration rates for experiments placed in the dark are presented in Figures 4.5 and 4.14 for TBT and atrazine. Tables 4.9 and 4.21 showed that there was no statistical significance that any treatment had an effect on the organism in the 6-h exposure experiment.

The 72-h cell count experiments for *E. gracilis* delivered inconclusive results. For both TBT (Figure 4.6) and atrazine (Figure 4.15) there was no significant difference between the reference and any of the treatments (Table 4.10 and 4.22 respectively for test chemicals). Using a regression curve linking cell numbers to absorbance readings poses a problem of overlooking the state of the cell and the organisms specific stress response might go unrecognized. *E. gracilis* under stressed conditions will change its shape from spindle to cyst form. The cell would still be present in the medium and the chloroplasts would still be sensed by a spectrophotometer. The cell count test did not accurately relay what was occurring in the samples. It is interesting to note that this same experiment did deliver significant results when performed with the green algae.

Cell morphology experiments over a 6-h period were investigated in the light and in the dark. Figure 4.7 (light) and Figure 4.8 (dark) showed the effects of TBT. Increased number of cyst cells ware present after a 6-h exposure to 10.0 µg/L and 100.0 µg/L of TBT and similar trends were observed in atrazine experiments in both 50.0 µg/L and 500.0 µg/L treatments. The carrier, 0.5% v/v DMSO, did not have significant effects on *E. gracilis* in any of the above mentioned experiments.

Respiration experiments for *E. gracilis* required large quantities of *Euglena* stock and also hours of preparation and set up prior to the actual experimental procedure. Following the 6-h, experiment the individual titrations were labour intensive and took approximately the same duration as the experiment itself. With this said, and the fact that no significant results were produced for this organism, respiration experiments did not deliver accurate representation of stressed conditions.

Cell shape responses occur very quickly and this endpoint can be automated. Time-lapse still photography or video analysis could be used to monitor *Euglena*'s behaviour over time. Computer software could be programmed to recognise a specific shape, either spindle (elongated) or cyst (round) (Figure 3.5), to determine the percentage of these different morphologies and calculate the percentage that poses a risk of significant toxicity. Also other parameters have been researched by Tahedl and Häder (1998) which can be automated for short term responses to many freshwater contaminants.

Anodonta grandis was initially observed for two parameters: valve behaviour (open/closed) and its respiration rate. Correlation between these two parameters would allow for identification of the overall effect of the test chemical. During experimentation, with this particular species of freshwater mussels, it was difficult to determine the configuration of the valves. It was decided that the results from the TBT exposure were inconclusive and behavioural experiments would not be repeated with this particular species of mussel. Mussels exposed to 0.1 µg/L of TBT showed an increasing trend of their respiration rate which indicates stressed behaviours. In the 1.0 and 10.0 µg/L of TBT treatments, there was a decreasing trend relative to the 0.1 µg/L of TBT treatment (Figure 4.9). When statistical analysis was conducted comparing the variance of the largest two treatments, it showed that there was no statistical difference in comparison with the reference.

Mussels exposed to 50.0 µg/L and 500.0 µg/L of atrazine displayed in Figure 4.18 showed significant differences in respiration rate, $p=0.007$ and $p<0.001$ respectively (Table 4.23). This 6-h respiration experiment showed that increased concentrations of atrazine clearly correlated with the reduced respiration rate for this freshwater mussel species. A DO probe was used for the atrazine tests and it delivered similar DO readings compared to the Winkler titrations performed for TBT tests. With this said, automation of mussel respiration experiments can be devised. The experimental design could be modified using multiple DO probes monitoring individual test vessels. A computer program would take DO readings periodically and compare test samples with reference vessels and determine a critical value that could deem a sample toxic. The behavioural experiments for this particular species of freshwater mussels did not deliver results that could be automated. Past experimental designs such as the "Dreissena-Monitor" developed by Borcherding and Volpers (1994) was designed to monitor open and closed alterations of different bi-valve species. This behaviour has proven to be a plausible endpoint for toxicity tests but *A. grandis* showed that its mechanical abilities do not allow for accurate analysis for this endpoint.

CHAPTER 5

CONCLUSIONS and RECOMMENDATIONS

The primary focus of this research was to select highly-sensitive aquatic indicator species that can detect contaminants and rapidly respond to unsuitable environmental conditions. The ability to actively monitor aquatic conditions with biological organisms delivers the advantage of real-time information (Gerhardt *et al.*, 2002; Häder, 2007). Assessments of water-source quality can be conducted in an ongoing manner and contamination could be identified rapidly. This biological system would deliver a holistic approach in detecting water conditions which would compliment current costly chemical water assessments. The selected indicator species, *Lemna minor*, *Pseudokirchneriella subcapitata*, *Euglena gracilis*, and *Anodonta grandis* demonstrated to be adequate species in behaving discriminately to varying concentrations TBT and atrazine.

L. minor proved to be a good indicator species for both TBT and atrazine. The growth inhibition experiments showed a significant decrease in growth rate at 10.0 µg/L of TBT and 500.0 µg/L atrazine. Also, *L. minor* growth inhibition experiments can be run at very low cost producing accurate results comparable to that of chemical analysis. The experimental set up proposed can also be automated with minimal additions. To decrease the response time it is recommended that *L. gibba*, another free-floating macrophyte with a doubling time of 0.7 days, be used to deliver quicker results.

Comparing all the tests conducted with *P. subcapitata*, the cell counts using the MultiScan microplate reader correlating OD at 650-nm with number of cells or chlorophyll content proved to be the best indicator of stress. These tests were conducted over a 72-h period but could be done in a shorter time frame. Significant differences occurred at 100.0 µg/L of TBT and 500.0 µg/L atrazine. This test required minimal materials, was cost effective relative to chemical analysis, and sampling times required

little time to produce results. It would be possible to construct a free-flow system for the experimental design of an early-warning biomonitoring system.

Behavioural analysis of *E. gracilis* observing the changes in the shape of the cell was the most effective test for this organism. The experiments delivered results in 6 hours which is relatively short-term compared to chemical analysis. The experimental design was very simple to construct but would require modification and computer programming to develop cell shape identification software. A large percentage of stressed organisms, identified by the cyst conformation, were identified in less than 6-h and at concentrations of 10.0 µg/L of TBT and 50.0 µg/L of atrazine in light experiments.

The freshwater mussels selected for the bioassays could not be monitored for valve movement behaviours due to the difficulties in identifying the open and closed configuration. Other researchers have used alternate species to monitor valve behaviours and have automated monitoring such behavioural characteristics. It is not recommended that this particular species of mussels, *A. grandis*, be used for behavioural experiments. Another disadvantage to using mollusc species is that they will accumulate certain chemicals in the soft tissues of their visceral mass and therefore can not be reused in further experiments. They must be placed in a holding tank after exposure to any chemical and new mussels must be used. In the field mussels will have to be replaced on a regular basis to monitor aquatic conditions. The respiration rates showed to be an accurate endpoint for tests conducted with TBT and atrazine. Significant changes in respiration rates were observed at 100.0 µg/L of TBT and 50.0 µg/L of atrazine. The experimental set up for *A. grandis* is quite simple and could be automated by increasing the number of DO probes used and also the development of a computer program to monitor the oxygen levels in test and reference samples. Recalibrating the DO probes and replacing the mussels would be the only ongoing maintenance required after the system was completely set up.

This research demonstrated that all organisms used in this study displayed sensitivity to selected concentrations of TBT and atrazine for specific endpoints and

allowed for accurate assessment of contaminants. Essentially, these laboratory tests will lead to the development of an automated biological system which can monitor a range of organisms' behaviours using computer analysis software to quantify these behavioural and biochemical endpoints to assess the quality of aquatic environments. A summary of currently-used technologies have been mentioned in this document, providing a starting point in the development of a holistic, multi-organism, multi-response, early-warning biomonitoring system. Only two chemicals were tested in this research and further experimentation with other chemical classes is recommended. Conducting tests with individual chemicals is not representative of a realistic aquatic environment. Analysis of one isolated chemicals is an important start to toxicity experiments to identify basic observations on an organism's response to stressed conditions. Performing the bioassays presented in this study using multiple chemicals at once, assessing the synergistic effects of multiple contaminants, would give insight to realistic scenarios in the field.

Monitoring the behaviours of living organisms delivers a realistic rapid assessment of unsuitable aquatic conditions and can provide the necessary information to reduce risk to end users. The development of this system will directly ameliorate the current methods used to test drinking water sources, providing awareness of unfit resources. This low-cost, real-time, holistic, biological early-warning system could benefit developing countries, where contaminated drinking water is of high concern. Also, this system could easily be used to assess industrial effluents from sewage treatment plants, oil sands refineries and runoff from agricultural lands.

References

Acero, J.L., Stemmler, K. and Von Gunten, U. 2000. Degradation kinetics of atrazine and its degradation products with ozone and OH radicals: A predictive tool for drinking water treatment. *Environmental Science and Technology*. 34 (4): 591-597.

Ackerman, F. 2007. The Economics of Atrazine. *International Journal of Occupational and Environmental Health*. 13(1): 437-445.

Allen, J.F. 2003. Cyclic, pseudocyclic and noncyclic photophosphorylation: new links in the chain. *Trends in Plant Sci.* 8(1):15 – 19.

Anderson, J. 2003. The environmental benefits of water recycling and reuse. *Water Science and Technology: Water Supply*. 3 (4): 1-10.

Antizar-Ladislao, B. 2008. Environmental levels, toxicity and human exposure to tributyltin (TBT)-contaminated marine environment. A review. *Environmental International.* 34: 292-308.

Aquatic Habitat Management. 2008. Algae: Selenastrum. Retrieved April 29, 2009. Available at: http://www.upi-usa.com/aquatics/Algae_Selenastrum.php

BIODIDAC. 2009a. Protista - Mastigophora – Euglenophyta. Retrieved April 29 2009. Available at: biodidac.bio.uottawa.ca/thumbnails/filedet.htm?File_name=PROT001BandFile_type=GIF

BIODIDAC. 2009b. Animalia - Mollusca - Bivalvia. Retrieved April 29 2009. Available at:biodidac.bio.uottawa.ca/thumbnails/filedet.htm?File_name=BIVA006BandFile_type=GIF

Bishop, W.E. and Perry, R.L. 1981. Development and Evaluation of a Flow-Through Growth Inhibition Test with Duckweed (*Lemna minor*). Aquatic Toxicity and Hazard Assessment: Forth Conference, ASTM STP 737. p.421 -435.

Borcherding, J. 2006. Ten years of practical experience with the Dreissena-Monitor, a biological early warning system for continuous water quality monitoring. *Hydrobiologia.* 556:417- 426.

Borcherding, J. and Volpers, M. 1994. The Dreissena-monitor –1st results on the application of this biological early warning system in the continuous monitoring of water quality. *Water Science and Technology*. 29: 199–201.

Brodkin, M.A., Madhoun, H., Rameswaran, M. and Vatnick, I. 2007. Atrazine is an immune disruptor in adult northern leopard frogs (*Rana pipiens*). *Environmental Toxicology and Chemistry*. 26(1): 80-84.

Byrne, R and McMahon, B. 1991. Acid-base and ionic regulation, during and following emersion, in the freshwater bivalve, *Anodonta grandis simpsoniana* (Bivalve: Unionidae). *Biological Bullitin.* (1): 289-297.

Caux, P.Y., Ménard, L. and Kent R. 1996. Comparative study of the Effects of MCPA, Butylate, Atrazine and Cyanazine on Selenastrum capricornutum. *Environmental Pollution.* 92(2):219-225.

Chan, L-Y., Heath, A.G. and Neves, R.J. 2001. Comparison of oxygen consumption in freshwater mussels (Unionidae) from different habitats during declining dissolve oxygen concentration. *Hydrobiologia*. 450: 209-214.

Checcuccgi, A. Colombetrti, G. and Lenci, F.F. 1976. Action Spectra for Photoaccumulation of Green and Colorless Euglena: Evidence For Identification of Receptor Pigments. *Photochemistry and Photobiology*. 23: 51-54.

Chèvre, N., Loepfe, C., Singer, H., Stamm, C., Fenner, K. and Escher, B.I. 2006. Including mixtures in the determination of water quality criteria for herbicides in surface water. *Environmental Science and Technology*. 40 (2): 426-435.

Chiavarini, S., Massanisso, P., Nicolai, P., Nobili, C. and Morabito, R. 2003. Butyltins concentration levels and imposex occurrence in snails from the Sicilian coasts (Italy). *Chemosphere*. 50: 311–319

Clark E.A., Sterritt, R.M. and Lester, J.N. 1988 The fate of tributyltin in the aquatic environment. Environ Sci Technol. 22:600-604.

Dorigo, U., Bourrain, X., Bérard, A. and Leboulanger, C. 2004. Seasonal changes in the sensitivity of river microalgae to atrazine and isoproturon along a contamination gradient. *Science of the Total Environment*. 318 (1-3): 101-114.

Dubey, S.K. and Roy, U. 2002. Review: Biodegradation of tributyltins (organotins) by marine bacteria. Applied Organometallic Chemistry. 17(1): 3-8.

Dugay, J.D., Miège, C. and Hennion, M.C. 1998. Effect of the various parameters governing solid-phase microextraction for the trace-determination of pesticides in water. J. Chrom A. 795(1): 27-42.

Environment Canada. 2007a. Biological Test Method:Test for Measuring the Inhibition of Growth Using the Freshwater Macrophyte, *Lemna minor*.

Environment Canada. 2007b. Biological Test Method: Growth Inhibition Test Using a Freshwater Algae. Retrieved May 1, 2008. Available at: http://www.etc-cte.ec.gc.ca/organization/bmd/pubs/pubs_en/RM252nded-AlgaeEnglish.pdf

Fairchild, J., Ruessler, D., Haverland, P. and Carlson, A. 1997. Comparative Sensitivity of *Selenastrum capricornutum* and *Lemna minor* to sixteen herbicides. *Arch. Environ. Contam. Toxicol.* 32: 353 – 357.

Fairchild, J., Ruessler, D., Haverland, P. and Carlson, A. 1998. Comparative Sensitivity of Five Species of Macrophytes and Six Species of Algae to Atrazine, Metribuzin, Alachlor, and Metolachlor. Environmental Toxicology and Chemistry. 17(9): 1830-1834.

Fent, K. 1996. Ecotoxicology of organotin compounds. *Crit Rev Toxicol.* 26: 3-117.

Gajdosova, J. and Reichrtova, E. 1996. Different growth response of *Euglena gracilis* to Hg, Cd, Cr and Ni compounds. *Fresenius J Anal Chem*. 354: 641 – 642.

Gerhardt, A., Jassens de Bisthoven, L. and Penders, E. 2003. Quality control of drinking water form the River Rhine with the Multispecies Freshwater Biomonitor. Aqua. *Ecosyst. Health and Manag*. 6(2):159 – 166.

Gerhardt, A., Schmidt, S. and Hoss, S. 2002. Measurment of movement patterns of *Caenorhabditis elegans* (Nematoda) with the Mulitspecies Freshwater Biomonitor® (MFB) – a potential new method to study a behavioural toxicity parameter of nematodes in sediments. *Enviro. Polut*. 120: 513 – 516.

Giesy, J.P. and Hoke R.A. 1989. Freshwater Sediment Toxicity Bioassessment: Rationale for Species Selection and Test Design. *J. of Great Lakes. Res.* 15(4): 539-569.

Giesy, J.P. and Allred, P.M. 1985. Replicability of aquatic multispecies test systems. *Multispecies Toxicity Testing*, J. Cairns Jr. ed. N.Y.: Pregamon Press. 14:187-247. Available at: http://www.usask.ca/toxicology/jgiesy/pdf/publications/BC-019.pdf

Gleick, P.H. 2000. The changing water paradigm a look at twenty-first century water resources development. *Water International*. 25 (1): 127-138.

Goodrich, J.A. and Lykins, B.W. 1991. Drinking Water from Agriculturally Contaminated Groundwater. Journal of Environmental Quality. 20:707-717.

Gunkel, G. and Streit, B. 1980. Mechanisms of bioaccumulation of a herbicide (atrazine, s-triazine) in a freshwater mollusc (Ancylus fluviatilis Mull.) and a fish (Coregonus fera Jurine). *Water Research*. 14 (11): 1573-1584

Häder, D.P. 2007. ECOTOX Biomonitoring pamphlet.

Health Canada. 1993. Atrazine Technical Document: Chemical/Physical Parameters. Retrieved 17 February, 2009. Available at: http://www.hc-sc.gc.ca/ewh-semt/pubs/water-eau/atrazine/index-eng.php

Hillman, W.S. 1961. The Lemnaceae, or Duckweeds: A Review of the Descriptive and Experimental Literature. *Botanical Review.* 27(2):221-287.

Hutner, S. H. 1953. Comparative physiology of heterotrophlc growth. In: W. E. Loomis (ed.), Growth and Differentiation of Plants. Iowa State College Press. pp. 417-446.

Johnson, L.E., Ricciardi, A. and Carlton, J.T. 2001. Overland dispersal of aquatic invasive species: A risk assessment of transient recreational boating. *Ecological Applications*. 11(6): 1789-1799.

Kaneko, H., Shimada, A. and Hirayama, K. 2004. Short-term algal toxicity test based on phosphate uptake. *Water Research*. 38(8):2173 – 2177.

Katsumata, M., Koike, T., Mishikawa, M., Kazumura, K., and Tsuchiya, H. 2006. Rapid ecotoxicological bioassay using delayed fluorescence in the green algae *Pseudokirchneriella subcapitata*. *Water Research*. 40: 3393 – 3400.

Kirkpatrick, A.J. Gerhardt, A., Dick, J.T.A., McKenna M. and Berges, J.A. 2006. Use of the multispecies freshwater biomonitor to assess behavioural changes of *Corophium volutator* (Pallas, 17766) (Crustacea, Amphipoda) in response to toxicant exposure in sediment. *Ecotox. And Enviro Safety*. 64: 298 – 303.

Kontrikla, A. 2009. Environmental management aspects for TBT antifouling wastes from the shipyards. *Journal of Environmental Management*. 90(SUPPL. 1): S77-S85.

Lachney, C.L. and Lonergan, T.A. 1985. Regulation in Cell Shape of *Euglena gracilis*. III. Involvement of Stable Microtubules. *Journal of Cell Science*. 74:219-237.

LIMCO International. 2008. Multispecies Freshwater Biomonitor® (MFB). Available at: www.limco-int.com/emfb.htm

Lonergan, T.A. and Williamson, L.C. 1988. Regulation in cell shape in *Euglena gracilis*. V. Time dependant responses to Ca^{2+} agonists and antagonists. *Journal of Cell Science*. 89:365-371.

Loucks, D.P. 2000. Sustainable water resources management. *Water International*. 25 (1): 3-10.

Luana, T.G., Jina, J., Chana, S.M.N, Wonga, Y.S. and Tama, N.F.Y. 2006. Biosorption and biodegradation of tributyltin (TBT) by alginate immobilized *Chlorella vulgaris* beads in several treatment cycles. Process Biochemistry. 41(7):1560-1565.

Mackie, G. L. 1991. Biology of the Exotic Zebra Mussel, Dreissena Polymorpha, in Relation to Native Bivalves and Its Potential Impact in Lake St. Clair. *Hydrobiologia*. 219(1): 251-268.

McCarthy, L. 2007. NSERC Grant Proposal.

Metcalfe, C.D., Miao, X.-S., Koenig, B.G. and Struger, J. 2003. Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. *Environmental Toxicology and Chemistry*. 22(12): 2881-2889.

Ministry of the Environment, Ontario. 2008a. Protocol of Accepted Drinking-Water Testing Methods. Retrieved March 10, 2009. Available at: http://www.ene.gov.on.ca/envision/gp/4465e01.pdf

Ministry of the Environment Drinking Water Ontario. 2008b. *Annual Report Template*. O. Reg. 170/03. Retrieved March 10, 2009. Available at: http://www.ontario.ca/drinkingwater/125607.pdf

Moody, M. and Miller, J. 2005. Small-scale Freshwater Toxicity Investigations: *Lemna minor* Growth Inhibition Tests. pp. 271-298.

Nikolaou, A.D., Gatidou, G.M., Golfinopoulos, S.K., Thomaidis, N. and Lekkas, T.D. 2007. A one-year survey of organotin compounds in the reservoirs supplying the drinking water treatment plants of Athens, Greece. *Desalination*. 210 (1-3): 24-30.

OECD. 2002a. OECD Guidelines for the testing of chemicals: revised proposal for a new guideline 221, Lemna sp. Growth Inhibition Test.

OECD. 2002b. OECD Guidelines for the Testing of Chemicals, Proposal for Updating Guideline 210, Freshwater Alga and Cyanobacteria, Growth Inhibition Test.

Ohta, M., Nakamura, K., Tsuchiya, H., Tazkma, K. and Suzki, T. 1999. Effect of Several Solutions Including Mineral-encaging Zeolites on the Restoration of Cell Motility of Tributyltin-intoxicated *Euglena gracilis* Z. *Bioscience, Biotechnology and Biochemistry*. 63 (10):1691-1696.

Prudham, S. 2004. Poisoning the well: Neoliberalism and the contamination of municipal water in Walkerton, Ontario. *Geoforum*. 35(3): 343-359.

Richards, A. 2005. The Walkerton Health Study. *The Canadian nurse*. 101(5): 16-21.

Sabik, H., Jeannotb R. and Rondeaua, B. 2000. Multiresidue methods using solid-phase extraction techniques for monitoring priority pesticides, including triazines and degradation products, in ground and surface waters. *J. of Chromatography A*. 885(1-2):217-236.

Safe Drinking Water Act. 2002. O. Reg. 169/03. Ontario Drinking Water Quality Standards. Retrieved February 15, 2009. Available at: http://www.e-laws.gov.on.ca/html/regs/english/elaws_regs_030169_e.htm

Sala, L. and Serra, M. 2004. Towards sustainability in water recycling. *Water Science and Technology*. 50 (2): 1-8

Streb, C., Richter, P., Ntefidou, M., Lebert, M. and Häder, D.P. 2002. ECOTOX – Biomonitoring Based on Real Time Movement Analysis of Unicellular Organisms. Proceedings of "Life on Earth", 8th European Symposium of Life Sciences Research in Space, 23rd Annual International Gravitational Physiology Meeting, Karolinska Institute, Stockholm, Sweeden. p. 241 – 242.

Slooff, W., de Zwart, D. and Marquenie, J.M. 1983. Detection limits of a biological monitoring system for chemical water pollution based on mussel activity. *Bulletin of Environmental Contamination and Toxicology*. 30(1): 400-405

Snyder, S.A., Westerhoff, P., Yoon, Y. and Sedlak, D.L. 2003. Pharmaceuticals, personal care products, and endocrine disruptors in water: Implications for the water industry. *Environmental Engineering Science*. 20 (5): 449-469.

Solomon, K.R., Baker, D.B., Richards, R.P., Dixon, K.R., Klaine, S.J. Thomas, La Point, W., Kendall, R.J, Weisskopf,C.P., Giddings, J.M., Giesy, J.P., Hall, L.W. Jr. and Williams, W.M. 1996. Ecological Risk Assessment of Atrazine in North American Surface Waters. *Environmental Toxicology and Chemistry*. 15 (1): 31–76.

Strehler, B.L. and Arnold W. 1951. Light Production by Green Plants. J Gen Physiol. 34(6): 809 – 820.

Tahedl, H. and Häder, D.P. 2001. Automated Biomonitoring Using Real Time Movement Analysis of *Euglena gracilis*. *Ecotox and Enviro Safety*. 48: 161 – 169.

Tahedl, H. and Häder, D.P. 1998. Fast Examination of Water Quality Using the Automatic Biotest ECOTOX Based on the Movement Behavior of a Freshwater Flagellate. *Wat. Res*. 33 (2): 426 – 432.

Texas Parks and Wildlife. 2008. Giant Floater (Anodonta grandis). Retrieved May 15 2008. Available at: www.tpwd.state.tx.us/huntwild/wild/species/giantfloater/

Topp, E., Monteiro, S.C., Beck, A., Coelho, B.B., Boxall, A.B.A, Duenk, P.W., Kleywegt, S., Lapen, D.R., Payne, M., Sabourin, L., Li, H. and Metcalfe, C.D. 2008. Runoff of pharmaceuticals and personal care products following application of biosolids to an agricultural field. *Science of The Total Environment*. 396(1): 52-59.

Tothill, I.E. and Turner, A.P.F. 1996. Developments in bioassay methods for toxicity testing in water treatment. *Trends in Analytical Chemistry.*15(5):178-188.

University of Florida. 2008. Center for Aquatic and Invasive Plants: Aquatic, Wetland and Invasive Plants Line Drawings. Retrieved June 9, 2009. Available at: http://plants.ifas.ufl.edu/images/line_drawings/lemmin2.jpg

USEPA. 1996a. Aquatic plant toxicity test using *Lemna* spp., Tiers I and II, Ecological Effects Test Guidelines OPPTS 850.4400

USEPA. 1996b. Algal Toxicity, Tiers I and II, Ecological Effects Test Guidelines OPPTS 850.5400

USEPA. 2003a. *Ambient Aquatic Life Water Quality Criteria for Tributyltin (TBT) – Final.* Retrieved February 12, 2009. Available at: http://www.epa.gov/waterscience/criteria/tributyltin/tbt-final.pdf

USEPA. 2003b. *Atrazine Interim Reregistration Eligibility Decision*. Retrieved February 14, 2009. Available at: http://www.epa.gov/pesticides/factsheets/atrazine.htm.

US Geological Survey. 1992, 2002. Pesticide National Synthesis Project. Retrieved 9 June 2009. Available at: http://water.usgs.gov/nawqa/pnsp/usage/maps/show_map.php?year=02andmap=m1980

van Slooten K.B. and Tarradellas, J. 1994. Accumulation, Depuration and Growth Effects of Tributyltin in the Freshwater Bivalve Dreissena polymorphia under field condiditons. *Enviro Tox and Chem*. 13 (5):755-762.

Wang, W. 1990. Literature Review on Duckweed Toxicity Testing. *Environmental Research*. 52: 7 – 22.

Wang, W. and Freemark, K. 1995. The Use of Plants for Environmental Monitoring and Assessment. *Ecotoxicological and Environmental Safety.* 30: 289-301.

Wraight C. A. and Crofts A. R. 1971. Delayed fluorescence and high-energy state of chloroplasts. Eur. J. Biochem. 19: 386 – 397.

Yang, F., Maguire, R.J. and Chau, Y.K. 2001. Occurrence of butyltin compounds in freshwater mussels (Elliptio complanata) from contaminated aquatic areas in Ontario, Canada. *Water Quality Research Journal of Canada*. 36(4): 805-814.

Yang, F. and Maguire R.J. 2000. Occurrence and seasonal variation of tributyltin in Marinas on Lake Ontario, Canada. *Water Quality Research Journal of Canada*. 35 (4): 681-691.

Appendix A: Ontario Drinking Water Quality Standards: O. Reg. 169/03 (Safe Drinking Water Act, 2002)

A. Microbiological Standards

Item	Microbiological Parameter	Standard (expressed as a maximum)
1.	*Escherichia coli* (*E. coli*)	Not detectable
2.	Total coliforms	Not detectable

B. Chemical Standards

Item	Chemical Parameter	Standard (expressed as a maximum concentration in milligrams per litre)
1.	Alachlor	0.005
2.	Aldicarb	0.009
3.	Aldrin + Dieldrin	0.0007
4.	Antimony	0.006
5.	Arsenic	0.025
6.	Atrazine + N-dealkylated metabolites	0.005
7.	Azinphos-methyl	0.02
8.	Barium	1.0
9.	Bendiocarb	0.04
10.	Benzene	0.005
11.	Benzo(a)pyrene	0.00001
12.	Boron	5.0
13.	Bromate	0.01
14.	Bromoxynil	0.005
15.	Cadmium	0.005
16.	Carbaryl	0.09
17.	Carbofuran	0.09
18.	Carbon Tetrachloride	0.005
19.	Chloramines	3.0
20.	Chlordane (Total)	0.007
21.	Chlorpyrifos	0.09
22.	Chromium	0.05
23.	Cyanazine	0.01
24.	Cyanide	0.2
25.	Diazinon	0.02
26.	Dicamba	0.12
27.	1,2-Dichlorobenzene	0.2
28.	1,4-Dichlorobenzene	0.005
29.	Dichlorodiphenyltrichloroethane (DDT) + metabolites	0.03
30.	1,2-dichloroethane	0.005
31.	1,1-Dichloroethylene (vinylidene chloride)	0.014
32.	Dichloromethane	0.05

33.	2,4-Dichlorophenol	0.9
34.	2,4-Dichlorophenoxy acetic acid (2,4-D)	0.1
35.	Diclofop-methyl	0.009
36.	Dimethoate	0.02
37.	Dinoseb	0.01
38.	Dioxin and Furan	0.000000015 [a]
39.	Diquat	0.07
40.	Diuron	0.15
41.	Fluoride	1.5
42.	Glyphosate	0.28
43.	Heptachlor + Heptachlor Epoxide	0.003
44.	Lead	0.010
45.	Lindane (Total)	0.004
46.	Malathion	0.19
47.	Mercury	0.001
48.	Methoxychlor	0.9
49.	Metolachlor	0.05
50.	Metribuzin	0.08
51.	Microcystin LR	0.0015
52.	Monochlorobenzene	0.08
53.	Nitrate (as nitrogen)	10.0
54.	Nitrite (as nitrogen)	1.0
55.	Nitrate + Nitrite (as nitrogen)	10.0
56.	Nitrilotriacetic Acid (NTA)	0.4
57.	N-Nitrosodimethylamine (NDMA)	0.000009
58.	Paraquat	0.01
59.	Parathion	0.05
60.	Pentachlorophenol	0.06
61.	Phorate	0.002
62.	Picloram	0.19
63.	Polychlorinated Biphenyls (PCB)	0.003
64.	Prometryne	0.001
65.	Selenium	0.01
66.	Simazine	0.01
67.	Temephos	0.28
68.	Terbufos	0.001
69.	Tetrachloroethylene (perchloroethylene)	0.03
70.	2,3,4,6-Tetrachlorophenol	0.1
71.	Triallate	0.23
72.	Trichloroethylene	0.005
73.	2,4,6-Trichlorophenol	0.005
74.	2,4,5-Trichlorophenoxy acetic acid (2,4,5-T)	0.28
75.	Trifluralin	0.045
76.	Trihalomethanes	0.100 [b]
77.	Uranium	0.02
78.	Vinyl Chloride	0.002

C. Radiological Standards

Item	Radiological Parameter	Standard (expressed as a maximum in becquerels per litre)
Natural Radionuclides		
1.	Beryllium-7	4000.0
2.	Bismuth -210	70.0
3.	Lead-210	0.1
4.	Polonium-210	0.2
5.	Radium-224	2.0
6.	Radium-226	0.6
7.	Radium-228	0.5
8.	Thorium-228	2.0
9.	Thorium-230	0.4
10.	Thorium-232	0.1
11.	Thorium-234	20.0
12.	Uranium-234	4.0
13.	Uranium-235	4.0
14.	Uranium-238	4.0
Artificial Radionuclides		
15.	Americium-241	0.2
16.	Antimony-122	50.0
17.	Antimony-124	40.0
18.	Antimony-125	100.0
19.	Barium-140	40.0
20.	Bromine-82	300.0
21.	Calcium-45	200.0
22.	Calcium-47	60.0
23.	Carbon-14	200.0
24.	Cerium-141	100.0
25.	Cerium-144	20.0
26.	Cesium-131	2000.0
27.	Cesium-134	7.0
28.	Cesium-136	50.0
29.	Cesium-137	10.0
30.	Chromium-51	3000.0
31.	Cobalt-57	40.0
32.	Cobalt-58	20.0
33.	Cobalt-60	2.0
34.	Gallium-67	500.0
35.	Gold-198	90.0
36.	Indium-111	400.0
37.	Iodine-125	10.0

38.	Iodine-129	1.0
39.	Iodine-131	6.0
40.	Iron-55	300.0
41.	Iron-59	40.0
42.	Manganese-54	200.0
43.	Mercury-197	400.0
44.	Mercury-203	80.0
45.	Molybdenum-99	70.0
46.	Neptunium-239	100.0
47.	Niobium-95	200.0
48.	Phosphorus-32	50.0
49.	Plutonium-238	0.3
50.	Plutonium-239	0.2
51.	Plutonium-240	0.2
52.	Plutonium-241	10.0
53.	Rhodium-105	300.0
54.	Rubidium-81	3000.0
55.	Rubidium-86	50.0
56.	Ruthenium-103	100.0
57.	Ruthenium-106	10.0
58.	Selenium-75	70.0
59.	Silver-108m	70.0
60.	Silver-110m	50.0
61.	Silver-111	70.0
62.	Sodium-22	50.0
63.	Strontium-85	300.0
64.	Strontium-89	40.0
65.	Strontium-90	5.0
66.	Sulphur-35	500.0
67.	Technetium-99	200.0
68.	Technetium-99m	7000.0
69.	Tellurium-129m	40.0
70.	Tellurium-131m	40.0
71.	Tellurium-132	40.0
72.	Thallium-201	2000.0
73.	Tritium	7000.0
74.	Ytterbium-169	100.0
75.	Yttrium-90	30.0
76.	Yttrium-91	30.0
77.	Zinc-65	40.0
78.	Zirconium-95	100.0

Appendix B: Sample Calculations

1. Winkler Titrations: Determining dissolved oxygen (DO) for 24.5 mL scintillation vial

a) Effective sample size

Total reagents added
0.5 mL $MnCl_2$ + 0.5 mL NaI/NaOH + 0.5 mL H_2SO_4 = 1.5 mL

b) Dilution factor

= vial volume (mL) / (vial volume (mL) + reagent volume (mL))
= 24.5 / (24.5 + 1.5) mL
= 0.942

c) Effective sample volume

= (vial volume) x (dilution factor)
= 24.5 mL x 0.942
= 23.079 mL

d) Dissolved oxygen (DO)

$$= \frac{\text{(titrant volume) x (3.58 µmol } O_2 \text{ / mL) (1000 mL / L)]}}{\text{Effective sample volume}}$$

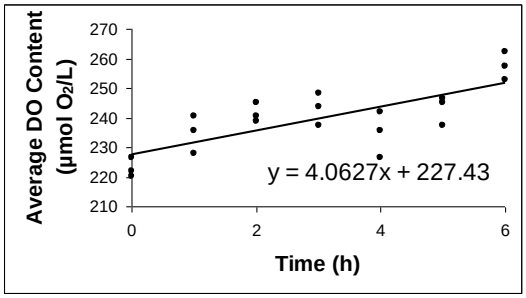
= titrant volume (155.1193) µmol O_2 / L

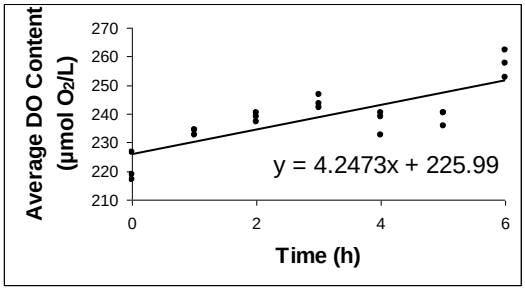
Appendix C: Raw Data for *Lemna minor* Growth Inhibition Experiments with Exposure to TBT.

		Ref			DMSO			TBT_1			TBT_10			TBT_100	
Time		1	2	3	1	2	3	1	2	3	1	2	3	1	2
	2 Fronds	5	5	5	5	5	5	5	5	5	5	5	5	5	5
0	3 Fronds	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4 Fronds	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Total Fronds	10	10	10	10	10	10	10	10	10	10	10	10	10	10
	1 Frond	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2 Fronds	5	3	3	3	4	2	2	1	4	2	2	3	5	5
24	3 Fronds	0	2	1	1	1	2	3	3	0	3	3	2	0	0
	4 Fronds	0	0	1	1	0	1	0	1	1	0	0	0	0	0
	Total Fronds	10	12	13	13	11	14	13	15	12	13	13	12	10	10
	1 Frond	0	0	0	0	0	0	0	0	0	0	0	0	1	1
	2 Fronds	3	1	1	1	3	1	2	0	3	2	2	2	0	0
48	3 Fronds	1	4	3	2	2	2	3	3	1	3	3	3	0	0
	4 Fronds	1	0	1	2	0	2	0	2	1	0	0	0	0	0
	Total Fronds	13	14	15	16	12	16	13	17	13	13	13	13	1	1
	1 Frond	0	0	0	0	0	0	0	0	0	0	0	0	0	0
72	2 Fronds	2	0	0	0	1	0	1	0	2	1	0	1	0	0
	3 Fronds	2	3	3	3	3	2	4	2	1	4	5	4	0	0
	4 Fronds	1	2	2	2	1	3	0	3	2	0	0	0	0	0
	Total Fronds	14	17	17	17	15	18	14	18	15	14	15	14	0	0
	1 Frond	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2 Fronds	1	0	0	0	0	0	1	0	1	0	0	1	0	0
96	3 Fronds	2	1	1	2	3	1	2	1	1	5	5	4	0	0
	4 Fronds	2	4	4	3	2	4	2	4	3	0	0	0	0	0
	Total Fronds	16	19	19	18	17	19	16	19	17	15	15	14	0	0

Appendix D: Raw Data for *Pseudokirchneriella subcapitata* Photosynthesis (Light) Experiments with exposure to TBT.

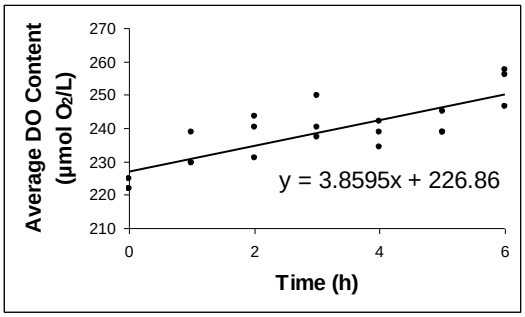
Reference			DMSO		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)	Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
REF 1 @ T0	1420	220.27	DMSO 1 @ T0	1410	218.72
REF 1 @ T1	1470	228.03	DMSO 1 @ T1	1500	232.68
REF 1 @ T2	1540	238.88	DMSO 1 @ T2	1540	238.88
REF 1 @ T3	1530	237.33	DMSO 1 @ T3	1560	241.99
REF 1 @ T4	1520	235.78	DMSO 1 @ T4	1500	232.68
REF 1 @ T5	1530	237.33	DMSO 1 @ T5	1520	235.78
REF 1 @ T6	1630	252.84	DMSO 1 @ T6	1630	252.84
REF 2 @ T0	1430	221.82	DMSO 2 @ T0	1400	217.17
REF 2 @ T1	1520	235.78	DMSO 2 @ T1	1510	234.23
REF 2 @ T2	1580	245.09	DMSO 2 @ T2	1530	237.33
REF 2 @ T3	1570	243.54	DMSO 2 @ T3	1570	243.54
REF 2 @ T4	1460	226.47	DMSO 2 @ T4	1550	240.44
REF 2 @ T5	1580	245.09	DMSO 2 @ T5	1550	240.44
REF 2 @ T6	1660	257.50	DMSO 2 @ T6	1660	257.50
REF 3 @ T0	1460	226.47	DMSO 3 @ T0	1460	226.47
REF 3 @ T1	1550	240.44	DMSO 3 @ T1	1510	234.23
REF 3 @ T2	1550	240.44	DMSO 3 @ T2	1550	240.44
REF 3 @ T3	1600	248.19	DMSO 3 @ T3	1590	246.64
REF 3 @ T4	1560	241.99	DMSO 3 @ T4	1540	238.88
REF 3 @ T5	1590	246.64	DMSO 3 @ T5	1550	240.44
REF 3 @ T6	1690	262.15	DMSO 3 @ T6	1690	262.15

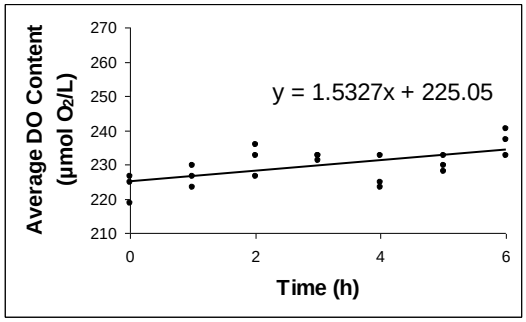




[TBT] = 10.0 µg/L		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
TBT 10.0 1 @ T0	1430	221.82
TBT 10.0 1 @ T1	1480	229.58
TBT 10.0 1 @ T2	1570	243.54
TBT 10.0 1 @ T3	1530	237.33
TBT 10.0 1 @ T4	1540	238.88
TBT 10.0 1 @ T5	1540	238.88
TBT 10.0 1 @ T6	1590	246.64
TBT 10.0 2 @ T0	1450	224.92
TBT 10.0 2 @ T1	1480	229.58
TBT 10.0 2 @ T2	1490	231.13
TBT 10.0 2 @ T3	1550	240.44
TBT 10.0 2 @ T4	1510	234.23
TBT 10.0 2 @ T5	1540	238.88
TBT 10.0 2 @ T6	1650	255.95
TBT 10.0 3 @ T0	1430	221.82
TBT 10.0 3 @ T1	1540	238.88
TBT 10.0 3 @ T2	1550	240.44
TBT 10.0 3 @ T3	1610	249.74
TBT 10.0 3 @ T4	1560	241.99
TBT 10.0 3 @ T5	1580	245.09
TBT 10.0 3 @ T6	1660	257.50

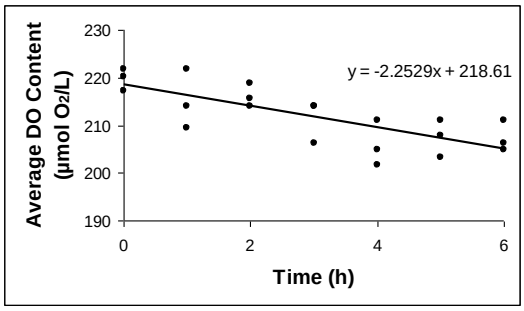
[TBT] = 100.0 µg/L		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
TBT 100.0 1 @ T0	1410	218.72
TBT 100.0 1 @ T1	1440	223.37
TBT 100.0 1 @ T2	1460	226.47
TBT 100.0 1 @ T3	1490	231.13
TBT 100.0 1 @ T4	1440	223.37
TBT 100.0 1 @ T5	1470	228.03
TBT 100.0 1 @ T6	1530	237.33
TBT 100.0 2 @ T0	1450	224.92
TBT 100.0 2 @ T1	1480	229.58
TBT 100.0 2 @ T2	1500	232.68
TBT 100.0 2 @ T3	1500	232.68
TBT 100.0 2 @ T4	1500	232.68
TBT 100.0 2 @ T5	1500	232.68
TBT 100.0 2 @ T6	1500	232.68
TBT 100.0 3 @ T0	1460	226.47
TBT 100.0 3 @ T1	1460	226.47
TBT 100.0 3 @ T2	1520	235.78
TBT 100.0 3 @ T3	1500	232.68
TBT 100.0 3 @ T4	1450	224.92
TBT 100.0 3 @ T5	1480	229.58
TBT 100.0 3 @ T6	1550	240.44

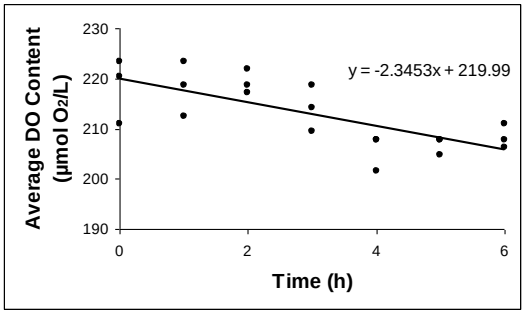




Appendix E: Raw Data for *Pseudokirchneriella subcapitata* Respiration (Dark) Experiments with Exposure to TBT.

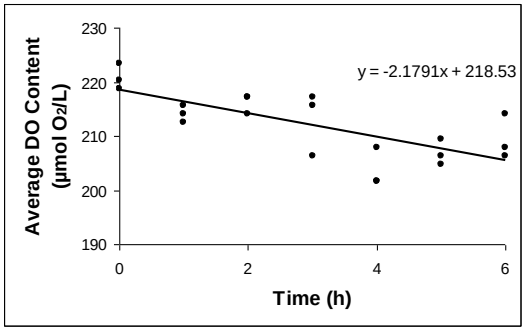
Reference			DMSO		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)	Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
REF 1 @ T0	1420	220.27	DMSO 1 @ T0	1420	220.27
REF 1 @ T1	1430	221.82	DMSO 1 @ T1	1410	218.72
REF 1 @ T2	1410	218.72	DMSO 1 @ T2	1430	221.82
REF 1 @ T3	1380	214.06	DMSO 1 @ T3	1380	214.06
REF 1 @ T4	1360	210.96	DMSO 1 @ T4	1340	207.86
REF 1 @ T5	1310	203.21	DMSO 1 @ T5	1320	204.76
REF 1 @ T6	1360	210.96	DMSO 1 @ T6	1360	210.96
REF 2 @ T0	1400	217.17	DMSO 2 @ T0	1360	210.96
REF 2 @ T1	1350	209.41	DMSO 2 @ T1	1370	212.51
REF 2 @ T2	1390	215.62	DMSO 2 @ T2	1400	217.17
REF 2 @ T3	1380	214.06	DMSO 2 @ T3	1410	218.72
REF 2 @ T4	1300	201.66	DMSO 2 @ T4	1300	201.66
REF 2 @ T5	1340	207.86	DMSO 2 @ T5	1340	207.86
REF 2 @ T6	1320	204.76	DMSO 2 @ T6	1330	206.31
REF 3 @ T0	1430	221.82	DMSO 3 @ T0	1440	223.37
REF 3 @ T1	1380	214.06	DMSO 3 @ T1	1440	223.37
REF 3 @ T2	1380	214.06	DMSO 3 @ T2	1410	218.72
REF 3 @ T3	1330	206.31	DMSO 3 @ T3	1350	209.41
REF 3 @ T4	1320	204.76	DMSO 3 @ T4	1340	207.86
REF 3 @ T5	1360	210.96	DMSO 3 @ T5	1340	207.86
REF 3 @ T6	1330	206.31	DMSO 3 @ T6	1340	207.86

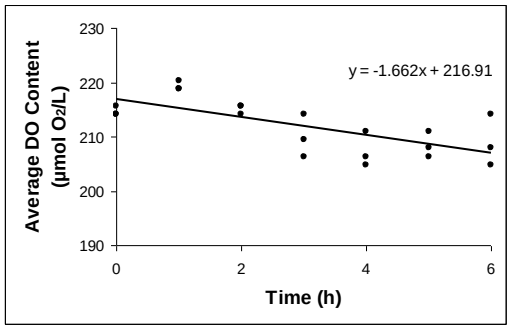




[TBT] = 10.0 µg/L		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
TBT 10.0 1 @ T0	1420	220.27
TBT 10.0 1 @ T1	1380	214.06
TBT 10.0 1 @ T2	1400	217.17
TBT 10.0 1 @ T3	1390	215.62
TBT 10.0 1 @ T4	1340	207.86
TBT 10.0 1 @ T5	1320	204.76
TBT 10.0 1 @ T6	1340	207.86
TBT 10.0 2 @ T0	1440	223.37
TBT 10.0 2 @ T1	1390	215.62
TBT 10.0 2 @ T2	1400	217.17
TBT 10.0 2 @ T3	1400	217.17
TBT 10.0 2 @ T4	1300	201.66
TBT 10.0 2 @ T5	1350	209.41
TBT 10.0 2 @ T6	1380	214.06
TBT 10.0 3 @ T0	1410	218.72
TBT 10.0 3 @ T1	1370	212.51
TBT 10.0 3 @ T2	1380	214.06
TBT 10.0 3 @ T3	1330	206.31
TBT 10.0 3 @ T4	1300	201.66
TBT 10.0 3 @ T5	1330	206.31
TBT 10.0 3 @ T6	1330	206.31

[TBT] = 100.0 µg/L		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
TBT 100.0 1 @ T0	1380	214.06
TBT 100.0 1 @ T1	1410	218.72
TBT 100.0 1 @ T2	1390	215.62
TBT 100.0 1 @ T3	1350	209.41
TBT 100.0 1 @ T4	1330	206.31
TBT 100.0 1 @ T5	1360	210.96
TBT 100.0 1 @ T6	1380	214.06
TBT 100.0 2 @ T0	1380	214.06
TBT 100.0 2 @ T1	1420	220.27
TBT 100.0 2 @ T2	1380	214.06
TBT 100.0 2 @ T3	1380	214.06
TBT 100.0 2 @ T4	1360	210.96
TBT 100.0 2 @ T5	1340	207.86
TBT 100.0 2 @ T6	1340	207.86
TBT 100.0 3 @ T0	1390	215.62
TBT 100.0 3 @ T1	1410	218.72
TBT 100.0 3 @ T2	1390	215.62
TBT 100.0 3 @ T3	1330	206.31
TBT 100.0 3 @ T4	1320	204.76
TBT 100.0 3 @ T5	1330	206.31
TBT 100.0 3 @ T6	1320	204.76





Appendix F: Raw Data for *Pseudokirchneriella subcapitata* Growth Inhibition Experiments with Exposure to TBT.

T=0	Number of Cells (x10^4)				
	1	2	3	Avg	Std (±)
Ref	371.9	373.8	375.7	373.8	1.9
DMSO	373.8	373.8	369.9	372.5	2.2
TBT_1	412.2	308.6	316.2	345.6	57.7
TBT_10	379.5	364.2	364.2	369.3	8.9
TBT_100	362.3	343.1	314.3	339.9	24.1

T=3	Number of Cells (x10^4)				
	1	2	3	Avg	Std (±)
Ref	408.3	427.5	408.3	414.7	11.1
DMSO	414.1	416.0	419.8	416.6	2.9
TBT_1	396.8	394.9	408.3	400.0	7.3
TBT_10	400.6	417.9	406.4	408.3	8.8
TBT_100	394.9	396.8	404.5	398.7	5.1

T=6	Number of Cells (x10^4)				
	1	2	3	Avg	Std (±)
Ref	406.4	417.9	400.6	408.3	8.8
DMSO	393.0	414.1	387.2	398.1	14.1
TBT_1	387.2	393.0	400.6	393.6	6.7
TBT_10	391.1	400.6	391.1	394.2	5.5
TBT_100	377.6	350.8	371.9	366.7	14.1

T=12	Number of Cells (x10^4)				
	1	2	3	Avg	Std (±)
Ref	414.1	421.7	393.0	409.6	14.9
DMSO	398.7	389.1	373.8	387.2	12.6
TBT_1	387.2	381.5	389.1	385.9	4.0
TBT_10	398.7	393.0	381.5	391.1	8.8
TBT_100	375.7	352.7	360.4	362.9	11.7

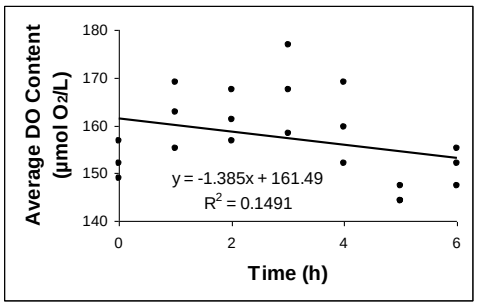
T=24	Number of Cells (x10^4)				
	1	2	3	Avg	Std (±)
Ref	540.7	540.7	594.4	558.6	31.0
DMSO	456.3	464.0	471.6	464.0	7.7
TBT_1	462.0	431.3	477.4	456.9	23.4
TBT_10	465.9	462.0	464.0	464.0	1.9
TBT_100	366.1	356.5	350.8	357.8	7.8

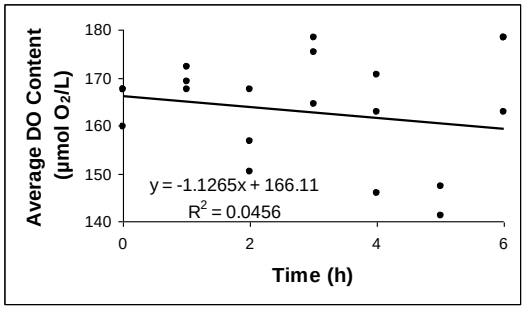
T=48	Number of Cells (x10^4)				
	1	2	3	Avg	Std (±)
Ref	1001.2	1083.6	1022.3	1035.7	42.9
DMSO	638.5	711.5	698.0	682.7	38.8
TBT_1	663.5	588.7	757.5	669.9	84.6
TBT_10	582.9	605.9	682.7	623.8	52.2
TBT_100	331.6	318.1	352.7	334.1	17.4

T=72	Number of Cells (x10^4)				
	1	2	3	Avg	Std (±)
Ref	1813.7	1789.7	2141.7	1915.0	196.7
DMSO	834.2	1021.3	1136.4	997.3	152.5
TBT_1	891.8	784.4	1125.9	934.0	174.6
TBT_10	809.3	901.4	1381.0	1030.6	307.0
TBT_100	323.9	287.4	368.0	326.5	40.4

Appendix G: Raw Data for *Euglena gracilis* Respiration Experiments in the Light with Exposure to TBT.

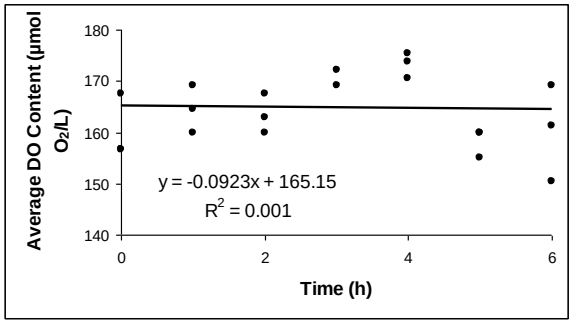
Reference			DMSO		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)	Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
REF 1 @ T0	980	152.02	DMSO 1 @ T0	1080	167.53
REF 1 @ T1	1000	155.12	DMSO 1 @ T1	1080	167.53
REF 1 @ T2	1010	156.67	DMSO 1 @ T2	970	150.47
REF 1 @ T3	1080	167.53	DMSO 1 @ T3	1150	178.39
REF 1 @ T4	980	152.02	DMSO 1 @ T4	940	145.81
REF 1 @ T5	930	144.26	DMSO 1 @ T5	860	133.40
REF 1 @ T6	980	152.02	DMSO 1 @ T6	1150	178.39
REF 2 @ T0	960	148.91	DMSO 2 @ T0	1080	167.53
REF 2 @ T1	1050	162.88	DMSO 2 @ T1	1110	172.18
REF 2 @ T2	1040	161.32	DMSO 2 @ T2	1080	167.53
REF 2 @ T3	1020	158.22	DMSO 2 @ T3	1060	164.43
REF 2 @ T4	1090	169.08	DMSO 2 @ T4	1100	170.63
REF 2 @ T5	930	144.26	DMSO 2 @ T5	950	147.36
REF 2 @ T6	1000	155.12	DMSO 2 @ T6	1150	178.39
REF 3 @ T0	1010	156.67	DMSO 3 @ T0	1030	159.77
REF 3 @ T1	1090	169.08	DMSO 3 @ T1	1090	169.08
REF 3 @ T2	1080	167.53	DMSO 3 @ T2	1010	156.67
REF 3 @ T3	1140	176.84	DMSO 3 @ T3	1130	175.28
REF 3 @ T4	1030	159.77	DMSO 3 @ T4	1050	162.88
REF 3 @ T5	950	147.36	DMSO 3 @ T5	910	141.16
REF 3 @ T6	950	147.36	DMSO 3 @ T6	1050	162.88

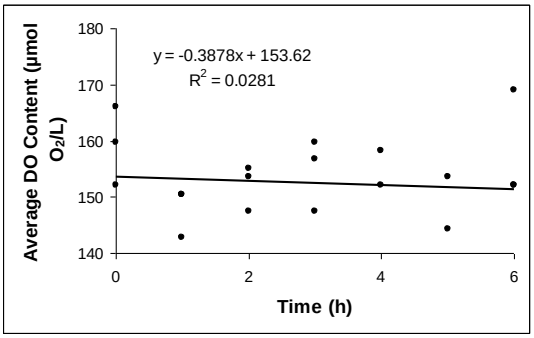




[TBT] = 10.0 µg/L		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
TBT 10.0 1 @ T0	1010	156.67
TBT 10.0 1 @ T1	1030	159.77
TBT 10.0 1 @ T2	1050	162.88
TBT 10.0 1 @ T3	1110	172.18
TBT 10.0 1 @ T4	1100	170.63
TBT 10.0 1 @ T5	1030	159.77
TBT 10.0 1 @ T6	970	150.47
TBT 10.0 2 @ T0	1010	156.67
TBT 10.0 2 @ T1	1060	164.43
TBT 10.0 2 @ T2	1030	159.77
TBT 10.0 2 @ T3	1090	169.08
TBT 10.0 2 @ T4	1120	173.73
TBT 10.0 2 @ T5	1030	159.77
TBT 10.0 2 @ T6	1040	161.32
TBT 10.0 3 @ T0	1080	167.53
TBT 10.0 3 @ T1	1090	169.08
TBT 10.0 3 @ T2	1080	167.53
TBT 10.0 3 @ T3	1170	181.49
TBT 10.0 3 @ T4	1130	175.28
TBT 10.0 3 @ T5	1000	155.12
TBT 10.0 3 @ T6	1090	169.08

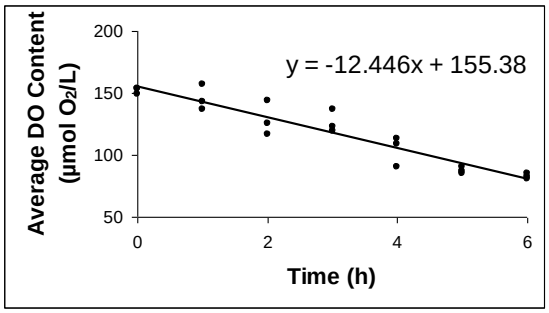
[TBT] = 100.0 µg/L		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
TBT 100.0 1 @ T0	1070	165.98
TBT 100.0 1 @ T1	920	142.71
TBT 100.0 1 @ T2	950	147.36
TBT 100.0 1 @ T3	950	147.36
TBT 100.0 1 @ T4	1020	158.22
TBT 100.0 1 @ T5	900	139.61
TBT 100.0 1 @ T6	1090	169.08
TBT 100.0 2 @ T0	1030	159.77
TBT 100.0 2 @ T1	970	150.47
TBT 100.0 2 @ T2	990	153.57
TBT 100.0 2 @ T3	1030	159.77
TBT 100.0 2 @ T4	980	152.02
TBT 100.0 2 @ T5	990	153.57
TBT 100.0 2 @ T6	980	152.02
TBT 100.0 3 @ T0	980	152.02
TBT 100.0 3 @ T1	970	150.47
TBT 100.0 3 @ T2	1000	155.12
TBT 100.0 3 @ T3	1010	156.67
TBT 100.0 3 @ T4	900	139.61
TBT 100.0 3 @ T5	930	144.26
TBT 100.0 3 @ T6	980	152.02

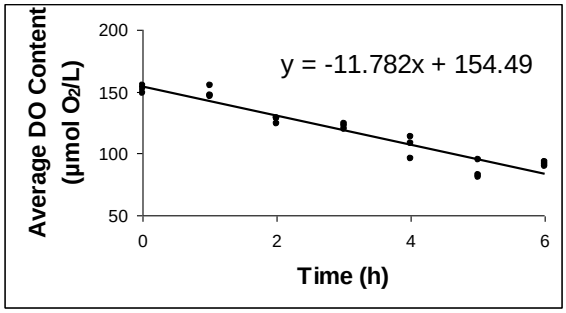




Appendix H: Raw Data for *Euglena gracilis* Respiration Experiments in the Dark with Exposure to TBT.

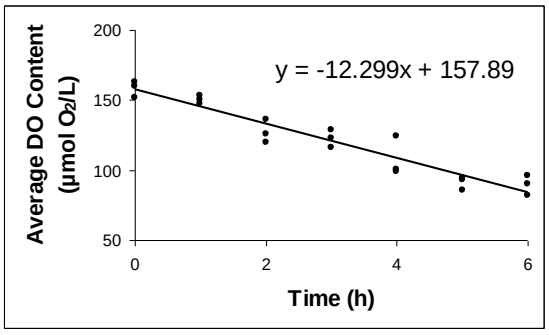
Reference			DMSO		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)	Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
REF 1 @ T0	990	153.57	DMSO 1 @ T0	1000	155.12
REF 1 @ T1	920	142.71	DMSO 1 @ T1	940	145.81
REF 1 @ T2	810	125.65	DMSO 1 @ T2	830	128.75
REF 1 @ T3	880	136.51	DMSO 1 @ T3	800	124.10
REF 1 @ T4	730	113.24	DMSO 1 @ T4	730	113.24
REF 1 @ T5	560	86.87	DMSO 1 @ T5	610	94.62
REF 1 @ T6	550	85.32	DMSO 1 @ T6	590	91.52
REF 2 @ T0	960	148.91	DMSO 2 @ T0	960	148.91
REF 2 @ T1	1010	156.67	DMSO 2 @ T1	950	147.36
REF 2 @ T2	930	144.26	DMSO 2 @ T2	830	128.75
REF 2 @ T3	790	122.54	DMSO 2 @ T3	770	119.44
REF 2 @ T4	580	89.97	DMSO 2 @ T4	700	108.58
REF 2 @ T5	580	89.97	DMSO 2 @ T5	530	82.21
REF 2 @ T6	520	80.66	DMSO 2 @ T6	580	89.97
REF 3 @ T0	990	153.57	DMSO 3 @ T0	980	152.02
REF 3 @ T1	880	136.51	DMSO 3 @ T1	1000	155.12
REF 3 @ T2	750	116.34	DMSO 3 @ T2	800	124.10
REF 3 @ T3	770	119.44	DMSO 3 @ T3	790	122.54
REF 3 @ T4	700	108.58	DMSO 3 @ T4	620	96.17
REF 3 @ T5	550	85.32	DMSO 3 @ T5	520	80.66
REF 3 @ T6	530	82.21	DMSO 3 @ T6	600	93.07

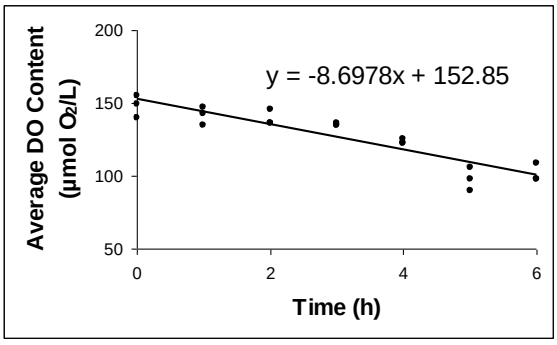




[TBT] = 10.0 µg/L		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
TBT 10.0 1 @ T0	1050	162.88
TBT 10.0 1 @ T1	950	147.36
TBT 10.0 1 @ T2	880	136.51
TBT 10.0 1 @ T3	790	122.54
TBT 10.0 1 @ T4	800	124.10
TBT 10.0 1 @ T5	600	93.07
TBT 10.0 1 @ T6	530	82.21
TBT 10.0 2 @ T0	1030	159.77
TBT 10.0 2 @ T1	990	153.57
TBT 10.0 2 @ T2	770	119.44
TBT 10.0 2 @ T3	750	116.34
TBT 10.0 2 @ T4	650	100.83
TBT 10.0 2 @ T5	610	94.62
TBT 10.0 2 @ T6	620	96.17
TBT 10.0 3 @ T0	980	152.02
TBT 10.0 3 @ T1	970	150.47
TBT 10.0 3 @ T2	810	125.65
TBT 10.0 3 @ T3	830	128.75
TBT 10.0 3 @ T4	640	99.28
TBT 10.0 3 @ T5	550	85.32
TBT 10.0 3 @ T6	580	89.97

[TBT] = 100.0 µg/L		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
TBT 100.0 1 @ T0	960	148.91
TBT 100.0 1 @ T1	920	142.71
TBT 100.0 1 @ T2	940	145.81
TBT 100.0 1 @ T3	870	134.95
TBT 100.0 1 @ T4	810	125.65
TBT 100.0 1 @ T5	630	97.73
TBT 100.0 1 @ T6	630	97.73
TBT 100.0 2 @ T0	900	139.61
TBT 100.0 2 @ T1	950	147.36
TBT 100.0 2 @ T2	880	136.51
TBT 100.0 2 @ T3	870	134.95
TBT 100.0 2 @ T4	790	122.54
TBT 100.0 2 @ T5	580	89.97
TBT 100.0 2 @ T6	700	108.58
TBT 100.0 3 @ T0	1000	155.12
TBT 100.0 3 @ T1	870	134.95
TBT 100.0 3 @ T2	880	136.51
TBT 100.0 3 @ T3	880	136.51
TBT 100.0 3 @ T4	790	122.54
TBT 100.0 3 @ T5	680	105.48
TBT 100.0 3 @ T6	630	97.73





Appendix I: Raw Data for *Euglena gracilis* Growth Inhibition Experiments with Exposure to TBT.

T=0	Number of Cells ($x10^4$)				
	1	2	3	Avg	Std (±)
Ref	14.4	13.0	13.2	13.5	0.78219
DMSO	16.1	15.3	15.5	15.6	0.40353
TBT_0.1	14.4	14.6	14.8	14.6	0.17331
TBT_1	15.5	16.2	15.9	15.9	0.38344
TBT_10	14.5	14.6	13.9	14.4	0.36442
TBT_100	15.9	16.2	13.9	15.3	1.27979

T=3	Number of Cells ($x10^4$)				
	1	2	3	Avg	Std (±)
Ref	13.5	14.6	14.7	14.3	0.63989
DMSO	19.0	17.5	17.1	17.9	1.02842
TBT_0.1	16.8	16.3	17.1	16.7	0.41512
TBT_1	18.9	17.7	17.9	18.1	0.66414
TBT_10	16.2	16.2	17.7	16.7	0.89529
TBT_100	21.8	21.3	16.9	20.0	2.69467

T=6	Number of Cells ($x10^4$)				
	1	2	3	Avg	Std (±)
Ref	14.3	12.4	13.6	13.4	0.97718
DMSO	17.2	17.5	17.0	17.2	0.27833
TBT_0.1	16.2	15.0	14.8	15.3	0.71900
TBT_1	16.6	15.0	14.7	15.5	1.02842
TBT_10	13.9	13.9	13.1	13.6	0.43737
TBT_100	20.4	17.7	15.9	18.0	2.25309

T=12	Number of Cells ($x10^4$)				
	1	2	3	Avg	Std (±)
Ref	18.1	18.4	20.0	18.8	1.02842
DMSO	23.6	21.5	21.2	22.1	1.30365
TBT_0.1	19.0	18.8	21.0	19.6	1.25673
TBT_1	22.1	20.9	20.8	21.3	0.73638
TBT_10	19.1	16.8	16.8	17.6	1.33244
TBT_100	25.9	25.0	21.4	24.1	2.36870

T=24	Number of Cells ($x10^4$)				
	1	2	3	Avg	Std (±)
Ref	29.9	25.5	25.0	26.8	2.65507
DMSO	27.5	23.7	26.1	25.8	1.91721
TBT_0.1	22.1	25.4	21.7	23.1	2.03831
TBT_1	37.3	29.7	34.6	33.9	3.84080
TBT_10	28.1	26.6	26.1	26.9	1.00352
TBT_100	36.3	28.1	25.7	30.1	5.55958

T=48	Number of Cells (x10^4)				
	1	2	3	Avg	Std (±)
Ref	58.7	44.9	45.1	49.6	7.91364
DMSO	50.7	46.0	49.6	48.7	2.40973
TBT_0.1	45.6	43.1	40.7	43.1	2.47925
TBT_1	53.2	47.3	50.7	50.4	2.97092
TBT_10	37.2	37.1	37.6	37.3	0.26073
TBT_100	52.9	44.2	35.9	44.3	8.50635

T=72	Number of Cells (x10^4)				
	1	2	3	Avg	Std (±)
Ref	99.6	83.5	99.4	94.2	9.24501
DMSO	76.1	72.1	95.0	81.0	12.25123
TBT_0.1	78.3	77.2	83.9	79.8	3.59590
TBT_1	88.3	96.3	111.6	98.7	11.85832
TBT_10	67.0	63.0	81.1	70.3	9.51602
TBT_100	117.9	84.5	79.3	93.9	20.91959

Appendix J: Raw Data for *Euglena gracilis* Behavioural Experiments in the Light with Exposure to TBT.

T=0	Number Spindle (Normal)			Number Cyst (Stressed)			Total			Percent Cyst (%)				
	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Avg	Rep1	Rep2	Rep3	Average percent cyst	Std
Reference	48	49	38	7	14	10	55	63	48	55	0.127	0.222	0.208	0.186	0.051
0.5% DMSO	66	77	90	9	11	11	75	88	101	88	0.120	0.125	0.109	0.118	0.008
10.0µg/L TBT	34	58	53	12	17	11	46	75	64	62	0.261	0.227	0.172	0.220	0.045
100.0µg/L TBT	50	32	28	8	18	6	58	50	34	47	0.138	0.360	0.176	0.225	0.119

T=3															
	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Avg	Rep1	Rep2	Rep3	Average percent cyst	Std
Reference	24	48	36	8	6	4	32	54	40	42	0.250	0.111	0.100	0.154	0.084
0.5% DMSO	22	28	31	1	4	6	23	32	37	31	0.043	0.125	0.162	0.110	0.061
10.0µg/L TBT	40	42	37	8	13	13	48	55	50	51	0.167	0.236	0.260	0.221	0.049
100.0µg/L TBT	42	50	47	9	16	11	51	66	58	58	0.176	0.242	0.190	0.203	0.035

T=6															
	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Avg	Rep1	Rep2	Rep3	Average percent cyst	Std
Reference	41	45	50	17	13	16	58	58	66	61	0.293	0.224	0.242	0.253	0.036
0.5% DMSO	60	35	31	18	12	7	78	47	38	54	0.231	0.255	0.184	0.223	0.036
10.0µg/L TBT	56	63	62	13	10	12	69	73	74	72	0.188	0.137	0.162	0.163	0.026
100.0µg/L TBT	33	37	43	19	13	23	52	50	66	56	0.365	0.260	0.348	0.325	0.057

T=24															
	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Avg	Rep1	Rep2	Rep3	Average percent cyst	Std
Reference	41	37	34	19	31	17	60	68	51	60	0.317	0.456	0.333	0.369	0.076
0.5% DMSO	63	35	53	40	19	37	103	54	90	82	0.388	0.352	0.411	0.384	0.030
10.0µg/L TBT	19	73	48	64	98	51	83	171	99	118	0.771	0.573	0.515	0.620	0.134
100.0µg/L TBT	22	24	41	35	36	44	57	60	85	67	0.614	0.600	0.518	0.577	0.052

Appendix K: Raw Data for *Euglena gracilis* Behavioural Experiments in the Dark with Exposure to TBT.

T=0	Number Spindle (Normal)			Number Cyst (Stressed)			Total				Percent Cyst (%)				
	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Avg	Rep1	Rep2	Rep3	Average percent cyst	Std (±)
Reference	29	13	29	3	1	2	32	14	31	26	0.094	0.071	0.065	0.077	0.015
0.5% DMSO	27	24	33	0	2	3	27	26	36	30	0.000	0.077	0.083	0.053	0.046
10.0µg/L TBT	78	51	78	10	7	3	88	58	81	76	0.114	0.121	0.037	0.090	0.046
100.0µg/L TBT	17	30	26	11	11	8	28	41	34	34	0.393	0.268	0.235	0.299	0.083

T=3															
	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Avg	Rep1	Rep2	Rep3	Average percent cyst	Std (±)
Reference	27	38	85	11	11	19	38	49	104	64	0.289	0.224	0.183	0.232	0.054
0.5% DMSO	22	37	40	12	6	6	34	43	46	41	0.353	0.140	0.130	0.208	0.126
10.0µg/L TBT	24	40	37	17	13	13	41	53	50	48	0.415	0.245	0.260	0.307	0.094
100.0µg/L TBT	81	76	60	11	23	16	92	99	76	89	0.120	0.232	0.211	0.187	0.060

T=6															
	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Avg	Rep1	Rep2	Rep3	Average percent cyst	Std (±)
Reference	31	38	34	28	24	28	59	62	62	61	0.475	0.387	0.452	0.438	0.045
0.5% DMSO	31	39	19	39	20	13	70	59	32	54	0.557	0.339	0.406	0.434	0.112
10.0µg/L TBT	17	25	35	15	10	20	32	35	55	41	0.469	0.286	0.364	0.373	0.092
100.0µg/L TBT	53	52	40	15	13	11	68	65	51	61	0.221	0.200	0.216	0.212	0.011

T=24															
	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Avg	Rep1	Rep2	Rep3	Average percent cyst	Std (±)
Reference	26	28	32	31	37	42	57	65	74	65	0.544	0.569	0.568	0.560	0.014
0.5% DMSO	16	21	32	25	50	43	41	71	75	62	0.610	0.704	0.573	0.629	0.068
10.0µg/L TBT	32	33	24	25	22	22	57	55	46	53	0.439	0.400	0.478	0.439	0.039
100.0µg/L TBT	26	49	22	13	10	12	39	59	34	44	0.333	0.169	0.353	0.285	0.101

Appendix L: Raw Data for *Anodonta grandis* Behavioural Experiments with Exposure to TBT.

Open = 0 Closed = 1

			Reference		
Time (hr)	1	2	3	4	5
0.0	1	1	1	1	1
1.0	1	0	0	1	1
2.0	0	1	1	1	1
3.0	0	1	1	1	1
4.0	0	1	0	1	1
5.0	0	1	1	1	1
6.0	0	1	0	1	1
			DMSO		
	1	2	3	4	5
0.0	1	1	1	1	0
1.0	1	1	0	1	1
2.0	1	1	0	1	1
3.0	1	1	0	1	0
4.0	0	0	0	0	0
5.0	0	0	0	1	1
6.0	0	0	0	0	0
			[TBT] = 0.1 µg/L		
	1	2	3	4	5
0.0	1	0	0	0	0
1.0	1	0	1	1	0
2.0	1	0	1	0	0
3.0	1	0	1	1	0
4.0	1	0	0	1	0
5.0	0	0	0	1	1
6.0	1	0	0	1	1
			[TBT] = 1.0 µg/L		
	1	2	3	4	5
0.0	1	1	1	0	1
1.0	1	1	0	1	1
2.0	1	1	0	1	1
3.0	1	0	0	0	1
4.0	1	0	0	1	0
5.0	1	0	1	0	0
6.0	1	0	0	0	1
			[TBT] = 10.0 µg/L		
	1	2	3	4	5
0.0	1	1	1	1	1
1.0	0	1	1	1	1
2.0	1	1	1	1	1
3.0	1	0	1	1	1
4.0	0	1	1	1	1
5.0	0	1	0	1	1
6.0	0	1	1	1	1

Appendix M: Raw Data for *Anodonta grandis* Respiration Experiments with Exposure to TBT.

Reference			DMSO		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)	Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
REF 1 @ T1	760	117.89	DMSO 1 @ T1	1230	190.80
REF 1 @ T2	1020	158.22	DMSO 1 @ T2	1260	195.45
REF 1 @ T3	850	131.85	DMSO 1 @ T3	1030	159.77
REF 1 @ T4	1010	156.67	DMSO 1 @ T4	1120	173.73
REF 1 @ T5	800	124.10	DMSO 1 @ T5	820	127.20
REF 1 @ T6	850	131.85	DMSO 1 @ T6	680	105.48
REF 1 @ T7	520	80.66	DMSO 1 @ T7	790	122.54
REF 2 @ T1	1240	192.35	DMSO 2 @ T1	1260	195.45
REF 2 @ T2	1240	192.35	DMSO 2 @ T2	1120	173.73
REF 2 @ T3	730	113.24	DMSO 2 @ T3	910	141.16
REF 2 @ T4	670	103.93	DMSO 2 @ T4	850	131.85
REF 2 @ T5	890	138.06	DMSO 2 @ T5	810	125.65
REF 2 @ T6	820	127.20	DMSO 2 @ T6	1300	201.66
REF 2 @ T7	1300	201.66	DMSO 2 @ T7	806	125.03
REF 3 @ T1	1140	176.84	DMSO 3 @ T1	850	131.85
REF 3 @ T2	1060	164.43	DMSO 3 @ T2	930	144.26
REF 3 @ T3	700	108.58	DMSO 3 @ T3	660	102.38
REF 3 @ T4	670	103.93	DMSO 3 @ T4	760	117.89
REF 3 @ T5	820	127.20	DMSO 3 @ T5	490	76.01
REF 3 @ T6	920	142.71	DMSO 3 @ T6	650	100.83
REF 3 @ T7	820	127.20	DMSO 3 @ T7	690	107.03
REF 4 @ T1	1170	181.49	DMSO 4 @ T1	1220	189.25
REF 4 @ T2	1160	179.94	DMSO 4 @ T2	1310	203.21
REF 4 @ T3	980	152.02	DMSO 4 @ T3	900	139.61
REF 4 @ T4	950	147.36	DMSO 4 @ T4	1270	197.00
REF 4 @ T5	910	141.16	DMSO 4 @ T5	1030	159.77
REF 4 @ T6	900	139.61	DMSO 4 @ T6	730	113.24
REF 4 @ T7	1020	158.22	DMSO 4 @ T7	980	152.02
REF 5 @ T1	1300	201.66	DMSO 5 @ T1	980	152.02
REF 5 @ T2	1310	203.21	DMSO 5 @ T2	1060	164.43
REF 5 @ T3	1180	183.04	DMSO 5 @ T3	550	85.32
REF 5 @ T4	970	150.47	DMSO 5 @ T4	1050	162.88
REF 5 @ T5	900	139.61	DMSO 5 @ T5	710	110.13
REF 5 @ T6	910	141.16	DMSO 5 @ T6	680	105.48
REF 5 @ T7	940	145.81	DMSO 5 @ T7	590	91.52

[TBT] = 0.1 µg/L			[TBT] = 1.0 µg/L		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)	Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
TBT 0.1 1 @ T1	1240	192.35	TBT 1.0 1 @ T1	1220	189.25
TBT 0.1 1 @ T2	920	142.71	TBT 1.0 1 @ T2	900	139.61
TBT 0.1 1 @ T3	930	144.26	TBT 1.0 1 @ T3	920	142.71
TBT 0.1 1 @ T4	400	62.05	TBT 1.0 1 @ T4	630	97.73
TBT 0.1 1 @ T5	690	107.03	TBT 1.0 1 @ T5	910	141.16
TBT 0.1 1 @ T6	360	55.84	TBT 1.0 1 @ T6	380	58.95
TBT 0.1 1 @ T7	860	133.40	TBT 1.0 1 @ T7	830	128.75
TBT 0.1 2 @ T1	1270	197.00	TBT 1.0 2 @ T1	1090	169.08
TBT 0.1 2 @ T2	1070	165.98	TBT 1.0 2 @ T2	1110	172.18
TBT 0.1 2 @ T3	750	116.34	TBT 1.0 2 @ T3	890	138.06
TBT 0.1 2 @ T4	1170	181.49	TBT 1.0 2 @ T4	630	97.73
TBT 0.1 2 @ T5	1180	183.04	TBT 1.0 2 @ T5	580	89.97
TBT 0.1 2 @ T6	750	116.34	TBT 1.0 2 @ T6	720	111.69
TBT 0.1 2 @ T7	660	102.38	TBT 1.0 2 @ T7	720	111.69
TBT 0.1 3 @ T1	1300	201.66	TBT 1.0 3 @ T1	1040	161.32
TBT 0.1 3 @ T2	1080	167.53	TBT 1.0 3 @ T2	930	144.26
TBT 0.1 3 @ T3	900	139.61	TBT 1.0 3 @ T3	860	133.40
TBT 0.1 3 @ T4	730	113.24	TBT 1.0 3 @ T4	960	148.91
TBT 0.1 3 @ T5	1110	172.18	TBT 1.0 3 @ T5	630	97.73
TBT 0.1 3 @ T6	1080	167.53	TBT 1.0 3 @ T6	420	65.15
TBT 0.1 3 @ T7	900	139.61	TBT 1.0 3 @ T7	550	85.32
TBT 0.1 4 @ T1	900	139.61	TBT 1.0 4 @ T1	1160	179.94
TBT 0.1 4 @ T2	1070	165.98	TBT 1.0 4 @ T2	1070	165.98
TBT 0.1 4 @ T3	830	128.75	TBT 1.0 4 @ T3	760	117.89
TBT 0.1 4 @ T4	900	139.61	TBT 1.0 4 @ T4	800	124.10
TBT 0.1 4 @ T5	640	99.28	TBT 1.0 4 @ T5	710	110.13
TBT 0.1 4 @ T6	460	71.35	TBT 1.0 4 @ T6	740	114.79
TBT 0.1 4 @ T7	610	94.62	TBT 1.0 4 @ T7	700	108.58
TBT 0.1 5 @ T1	1250	193.90	TBT 1.0 5 @ T1	1130	175.28
TBT 0.1 5 @ T2	1160	179.94	TBT 1.0 5 @ T2	850	131.85
TBT 0.1 5 @ T3	890	138.06	TBT 1.0 5 @ T3	620	96.17
TBT 0.1 5 @ T4	640	99.28	TBT 1.0 5 @ T4	730	113.24
TBT 0.1 5 @ T5	630	97.73	TBT 1.0 5 @ T5	450	69.80
TBT 0.1 5 @ T6	890	138.06	TBT 1.0 5 @ T6	940	145.81
TBT 0.1 5 @ T7	720	111.69	TBT 1.0 5 @ T7	800	124.10

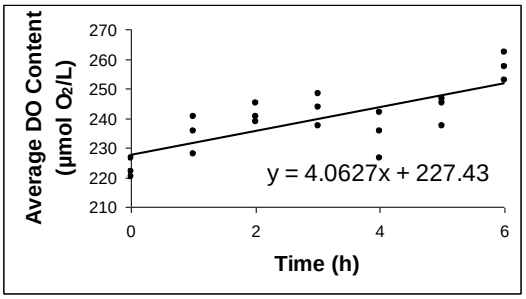
[TBT] = 10.0 µg/L		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
TBT 10.0 1 @ T1	1300	201.66
TBT 10.0 1 @ T2	1050	162.88
TBT 10.0 1 @ T3	820	127.20
TBT 10.0 1 @ T4	770	119.44
TBT 10.0 1 @ T5	790	122.54
TBT 10.0 1 @ T6	810	125.65
TBT 10.0 1 @ T7	730	113.24
TBT 10.0 2 @ T1	1320	204.76
TBT 10.0 2 @ T2	670	103.93
TBT 10.0 2 @ T3	860	133.40
TBT 10.0 2 @ T4	850	131.85
TBT 10.0 2 @ T5	500	77.56
TBT 10.0 2 @ T6	1090	169.08
TBT 10.0 2 @ T7	910	141.16
TBT 10.0 3 @ T1	1090	169.08
TBT 10.0 3 @ T2	1060	164.43
TBT 10.0 3 @ T3	620	96.17
TBT 10.0 3 @ T4	600	93.07
TBT 10.0 3 @ T5	790	122.54
TBT 10.0 3 @ T6	580	89.97
TBT 10.0 3 @ T7	810	125.65
TBT 10.0 4 @ T1	960	148.91
TBT 10.0 4 @ T2	1180	183.04
TBT 10.0 4 @ T3	1230	190.80
TBT 10.0 4 @ T4	480	74.46
TBT 10.0 4 @ T5	770	119.44
TBT 10.0 4 @ T6	930	144.26
TBT 10.0 4 @ T7	750	116.34
TBT 10.0 5 @ T1	1300	201.66
TBT 10.0 5 @ T2	840	130.30
TBT 10.0 5 @ T3	970	150.47
TBT 10.0 5 @ T4	960	148.91
TBT 10.0 5 @ T5	700	108.58
TBT 10.0 5 @ T6	1230	190.80
TBT 10.0 5 @ T7	1010	156.67

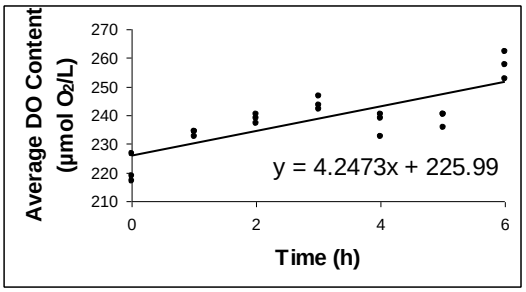
Appendix N: Raw Data for *Lemna minor* Growth Inhibition Experiments with Exposure to Atrazine.

		Ref			DMSO			ATZ_5			ATZ_50			ATZ_500		
Time		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
	2 Fronds	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
0	3 Fronds	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4 Fronds	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Total Fronds	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
	1 Frond	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2 Fronds	5	3	3	3	4	2	4	5	3	4	4	1	1	4	3
24	3 Fronds	0	2	1	1	1	2	1	0	2	1	1	4	4	1	2
	4 Fronds	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0
	Total Fronds	10	12	13	13	11	14	11	10	12	11	11	14	14	11	12
	1 Frond	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	2 Fronds	3	1	1	1	3	1	3	3	1	2	2	1	2	4	0
48	3 Fronds	1	4	3	2	2	2	2	2	4	2	2	4	3	1	3
	4 Fronds	1	0	1	2	0	2	0	0	0	1	1	0	0		0
	Total Fronds	13	14	15	16	12	16	12	12	14	14	14	14	13	11	10
	1 Frond	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
72	2 Fronds	2	0	0	0	1	0	2	2	0	2	0	2	2	3	0
	3 Fronds	2	3	3	3	3	2	3	2	3	1	4	3	3	2	3
	4 Fronds	1	2	2	2	1	3	0	0	2	2	1	0	0	0	0
	Total Fronds	14	17	17	17	15	18	13	11	17	15	16	13	13	12	10
	1 Frond	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0
	2 Fronds	1	0	0	0	0	0	1	1	0	1	0	1	1	2	1
96	3 Fronds	2	1	1	2	3	1	3	2	3	2	3	4	3	3	3
	4 Fronds	2	4	4	3	2	4	1	1	2	2	2	0	0	0	0
	Total Fronds	16	19	19	18	17	19	15	13	17	16	17	14	12	13	11

Appendix O: Raw Data for *Pseudokirchneriella subcapitata* Photosynthesis (Light) Experiments with Exposure to Atrazine.

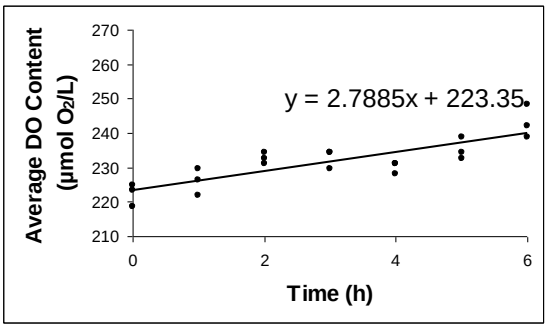
Reference			DMSO		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)	Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
REF 1 @ T0	1420	220.27	DMSO 1 @ T0	1410	218.72
REF 1 @ T1	1470	228.03	DMSO 1 @ T1	1500	232.68
REF 1 @ T2	1540	238.88	DMSO 1 @ T2	1540	238.88
REF 1 @ T3	1530	237.33	DMSO 1 @ T3	1560	241.99
REF 1 @ T4	1520	235.78	DMSO 1 @ T4	1500	232.68
REF 1 @ T5	1530	237.33	DMSO 1 @ T5	1520	235.78
REF 1 @ T6	1630	252.84	DMSO 1 @ T6	1630	252.84
REF 2 @ T0	1430	221.82	DMSO 2 @ T0	1400	217.17
REF 2 @ T1	1520	235.78	DMSO 2 @ T1	1510	234.23
REF 2 @ T2	1580	245.09	DMSO 2 @ T2	1530	237.33
REF 2 @ T3	1570	243.54	DMSO 2 @ T3	1570	243.54
REF 2 @ T4	1460	226.47	DMSO 2 @ T4	1550	240.44
REF 2 @ T5	1580	245.09	DMSO 2 @ T5	1550	240.44
REF 2 @ T6	1660	257.50	DMSO 2 @ T6	1660	257.50
REF 3 @ T0	1460	226.47	DMSO 3 @ T0	1460	226.47
REF 3 @ T1	1550	240.44	DMSO 3 @ T1	1510	234.23
REF 3 @ T2	1550	240.44	DMSO 3 @ T2	1550	240.44
REF 3 @ T3	1600	248.19	DMSO 3 @ T3	1590	246.64
REF 3 @ T4	1560	241.99	DMSO 3 @ T4	1540	238.88
REF 3 @ T5	1590	246.64	DMSO 3 @ T5	1550	240.44
REF 3 @ T6	1690	262.15	DMSO 3 @ T6	1690	262.15

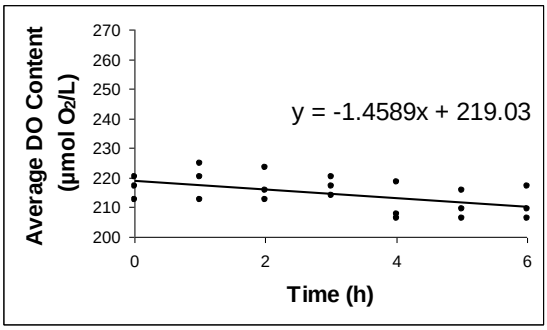




[Atrazine] = 50.0 µg/L		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
ATZ 50.0 1 @ T0	1440	223.37
ATZ 50.0 1 @ T1	1430	221.82
ATZ 50.0 1 @ T2	1500	232.68
ATZ 50.0 1 @ T3	1480	229.58
ATZ 50.0 1 @ T4	1470	228.03
ATZ 50.0 1 @ T5	1500	232.68
ATZ 50.0 1 @ T6	1540	238.88
ATZ 50.0 2 @ T0	1410	218.72
ATZ 50.0 2 @ T1	1460	226.47
ATZ 50.0 2 @ T2	1510	234.23
ATZ 50.0 2 @ T3	1510	234.23
ATZ 50.0 2 @ T4	1490	231.13
ATZ 50.0 2 @ T5	1540	238.88
ATZ 50.0 2 @ T6	1600	248.19
ATZ 50.0 3 @ T0	1450	224.92
ATZ 50.0 3 @ T1	1480	229.58
ATZ 50.0 3 @ T2	1490	231.13
ATZ 50.0 3 @ T3	1510	234.23
ATZ 50.0 3 @ T4	1490	231.13
ATZ 50.0 3 @ T5	1510	234.23
ATZ 50.0 3 @ T6	1560	241.99

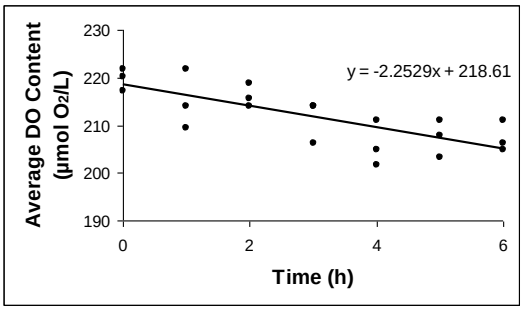
[TBT] = 500.0 µg/L		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
ATZ 500.0 1 @ T0	1370	212.51
ATZ 500.0 1 @ T1	1370	212.51
ATZ 500.0 1 @ T2	1370	212.51
ATZ 500.0 1 @ T3	1380	214.06
ATZ 500.0 1 @ T4	1340	207.86
ATZ 500.0 1 @ T5	1350	209.41
ATZ 500.0 1 @ T6	1350	209.41
ATZ 500.0 2 @ T0	1420	220.27
ATZ 500.0 2 @ T1	1420	220.27
ATZ 500.0 2 @ T2	1390	215.62
ATZ 500.0 2 @ T3	1400	217.17
ATZ 500.0 2 @ T4	1330	206.31
ATZ 500.0 2 @ T5	1330	206.31
ATZ 500.0 2 @ T6	1330	206.31
ATZ 500.0 3 @ T0	1400	217.17
ATZ 500.0 3 @ T1	1450	224.92
ATZ 500.0 3 @ T2	1440	223.37
ATZ 500.0 3 @ T3	1420	220.27
ATZ 500.0 3 @ T4	1410	218.72
ATZ 500.0 3 @ T5	1390	215.62
ATZ 500.0 3 @ T6	1400	217.17

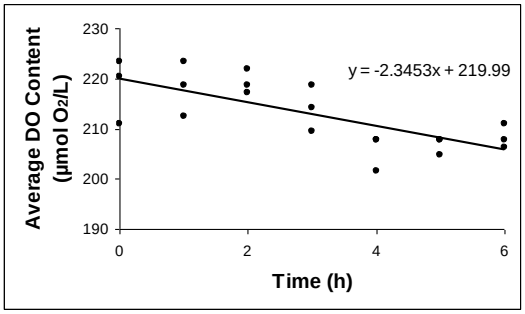




Appendix P: Raw Data for *Pseudokirchneriella subcapitata* Respiration Experiments with Exposure to Atrazine.

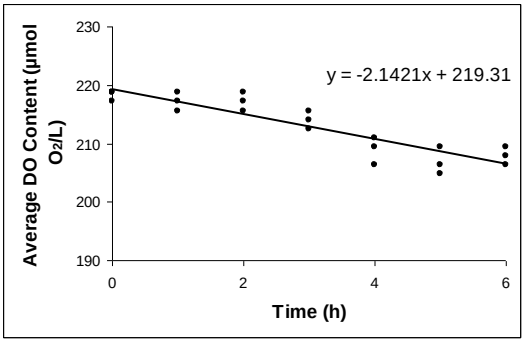
Reference			DMSO		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)	Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
REF 1 @ T0	1420	220.27	DMSO 1 @ T0	1420	220.27
REF 1 @ T1	1430	221.82	DMSO 1 @ T1	1410	218.72
REF 1 @ T2	1410	218.72	DMSO 1 @ T2	1430	221.82
REF 1 @ T3	1380	214.06	DMSO 1 @ T3	1380	214.06
REF 1 @ T4	1360	210.96	DMSO 1 @ T4	1340	207.86
REF 1 @ T5	1310	203.21	DMSO 1 @ T5	1320	204.76
REF 1 @ T6	1360	210.96	DMSO 1 @ T6	1360	210.96
REF 2 @ T0	1400	217.17	DMSO 2 @ T0	1360	210.96
REF 2 @ T1	1350	209.41	DMSO 2 @ T1	1370	212.51
REF 2 @ T2	1390	215.62	DMSO 2 @ T2	1400	217.17
REF 2 @ T3	1380	214.06	DMSO 2 @ T3	1410	218.72
REF 2 @ T4	1300	201.66	DMSO 2 @ T4	1300	201.66
REF 2 @ T5	1340	207.86	DMSO 2 @ T5	1340	207.86
REF 2 @ T6	1320	204.76	DMSO 2 @ T6	1330	206.31
REF 3 @ T0	1430	221.82	DMSO 3 @ T0	1440	223.37
REF 3 @ T1	1380	214.06	DMSO 3 @ T1	1440	223.37
REF 3 @ T2	1380	214.06	DMSO 3 @ T2	1410	218.72
REF 3 @ T3	1330	206.31	DMSO 3 @ T3	1350	209.41
REF 3 @ T4	1320	204.76	DMSO 3 @ T4	1340	207.86
REF 3 @ T5	1360	210.96	DMSO 3 @ T5	1340	207.86
REF 3 @ T6	1330	206.31	DMSO 3 @ T6	1340	207.86

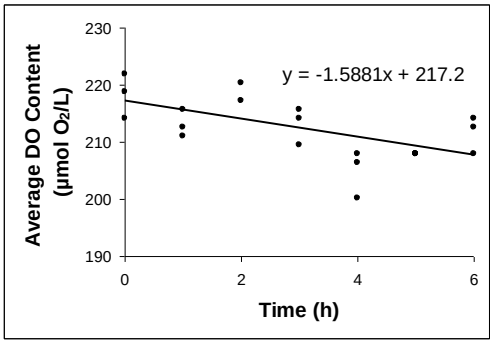




[Atrazine] = 50.0 µg/L		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
ATZ 50.0 1 @ T0	1410	218.72
ATZ 50.0 1 @ T1	1400	217.17
ATZ 50.0 1 @ T2	1400	217.17
ATZ 50.0 1 @ T3	1390	215.62
ATZ 50.0 1 @ T4	1350	209.41
ATZ 50.0 1 @ T5	1330	206.31
ATZ 50.0 1 @ T6	1330	206.31
ATZ 50.0 1 @ T0	1400	217.17
ATZ 50.0 1 @ T1	1410	218.72
ATZ 50.0 1 @ T2	1390	215.62
ATZ 50.0 1 @ T3	1380	214.06
ATZ 50.0 1 @ T4	1360	210.96
ATZ 50.0 1 @ T5	1350	209.41
ATZ 50.0 1 @ T6	1350	209.41
ATZ 50.0 1 @ T0	1410	218.72
ATZ 50.0 1 @ T1	1390	215.62
ATZ 50.0 1 @ T2	1410	218.72
ATZ 50.0 1 @ T3	1370	212.51
ATZ 50.0 1 @ T4	1330	206.31
ATZ 50.0 1 @ T5	1320	204.76
ATZ 50.0 1 @ T6	1340	207.86

[Atrazine] = 500.0 µg/L		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
ATZ 500.0 1 @ T0	1380	214.06
ATZ 500.0 1 @ T1	1390	215.62
ATZ 500.0 1 @ T2	1420	220.27
ATZ 500.0 1 @ T3	1380	214.06
ATZ 500.0 1 @ T4	1290	200.10
ATZ 500.0 1 @ T5	1340	207.86
ATZ 500.0 1 @ T6	1370	212.51
ATZ 500.0 1 @ T0	1430	221.82
ATZ 500.0 1 @ T1	1370	212.51
ATZ 500.0 1 @ T2	1400	217.17
ATZ 500.0 1 @ T3	1390	215.62
ATZ 500.0 1 @ T4	1330	206.31
ATZ 500.0 1 @ T5	1340	207.86
ATZ 500.0 1 @ T6	1380	214.06
ATZ 500.0 1 @ T0	1410	218.72
ATZ 500.0 1 @ T1	1360	210.96
ATZ 500.0 1 @ T2		
ATZ 500.0 1 @ T3	1350	209.41
ATZ 500.0 1 @ T4	1340	207.86
ATZ 500.0 1 @ T5	1340	207.86
ATZ 500.0 1 @ T6	1340	207.86





Appendix Q: Raw Data for *Pseudokirchneriella subcapitata* Growth Inhibition Experiments with Exposure to Atrazine.

T=0	Number of Cells (x10^4)			
	1	2	3	Avg
Ref	371.9	373.8	375.7	373.8
DMSO	373.8	373.8	369.9	372.5
ATZ_5	396.8	393.0	341.2	377.0
ATZ_50	364.2	335.4	283.6	327.7
ATZ_500	368.0	331.6	302.8	334.1

T=3	Number of Cells (x10^4)			
	1	2	3	Avg
Ref	408.3	427.5	408.3	414.7
DMSO	414.1	416.0	419.8	416.6
ATZ_5	394.9	402.6	429.4	409.0
ATZ_50	366.1	398.7	220.3	328.4
ATZ_500	383.4	383.4	385.3	384.0

T=6	Number of Cells (x10^4)			
	1	2	3	Avg
Ref	406.4	417.9	400.6	408.3
DMSO	393.0	414.1	387.2	398.1
ATZ_5	393.0	362.3	381.5	378.9
ATZ_50	360.4	366.1	364.2	363.6
ATZ_500	375.7	357.5	352.7	362.0

T=12	Number of Cells (x10^4)			
	1	2	3	Avg
Ref	414.1	421.7	393.0	409.6
DMSO	398.7	389.1	373.8	387.2
ATZ_5	396.8	373.8	379.5	383.4
ATZ_50	352.7	339.3	352.7	348.2
ATZ_500	364.2	346.2	337.3	349.2

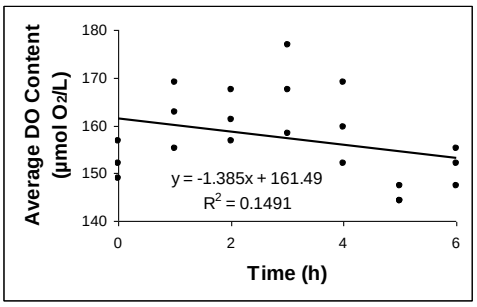
T=24	Number of Cells (x10^4)			
	1	2	3	Avg
Ref	540.7	540.7	594.4	558.6
DMSO	456.3	464.0	471.6	464.0
ATZ_5	498.5	446.7	446.7	464.0
ATZ_50	429.4	417.9	396.8	414.7
ATZ_500	369.9	358.0	362.3	363.4

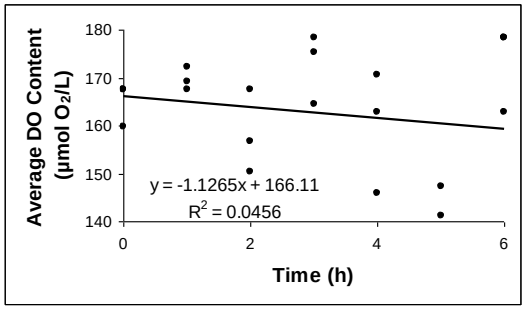
T=24	Number of Cells (x10^4)			
	1	2	3	Avg
Ref	1001.2	1083.6	1022.3	1035.7
DMSO	638.5	711.5	698.0	682.7
ATZ_5	826.6	684.6	659.6	723.6
ATZ_50	575.2	584.8	609.8	589.9
ATZ_500	354.6	334.0	337.3	342.0

T=72	Number of Cells ($x10^4$)			
	1	2	3	Avg
Ref	1813.7	1789.7	2141.7	1915.0
DMSO	834.2	1021.3	1136.4	997.3
ATZ_5	872.6	1071.2	866.9	936.9
ATZ_50	648.1	684.6	711.5	681.4
ATZ_500	362.3	427.0	412.2	400.5

Appendix R: Raw Data for *Euglena gracilis* Respiration Experiments in the Light with Exposure to Atrazine.

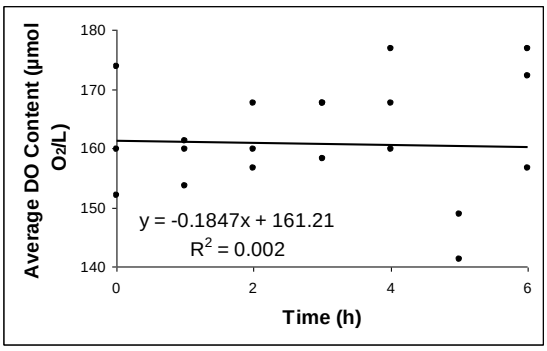
Reference			DMSO		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)	Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
REF 1 @ T0	980	152.02	DMSO 1 @ T0	1080	167.53
REF 1 @ T1	1000	155.12	DMSO 1 @ T1	1080	167.53
REF 1 @ T2	1010	156.67	DMSO 1 @ T2	970	150.47
REF 1 @ T3	1080	167.53	DMSO 1 @ T3	1150	178.39
REF 1 @ T4	980	152.02	DMSO 1 @ T4	940	145.81
REF 1 @ T5	930	144.26	DMSO 1 @ T5	860	133.40
REF 1 @ T6	980	152.02	DMSO 1 @ T6	1150	178.39
REF 2 @ T0	960	148.91	DMSO 2 @ T0	1080	167.53
REF 2 @ T1	1050	162.88	DMSO 2 @ T1	1110	172.18
REF 2 @ T2	1040	161.32	DMSO 2 @ T2	1080	167.53
REF 2 @ T3	1020	158.22	DMSO 2 @ T3	1060	164.43
REF 2 @ T4	1090	169.08	DMSO 2 @ T4	1100	170.63
REF 2 @ T5	930	144.26	DMSO 2 @ T5	950	147.36
REF 2 @ T6	1000	155.12	DMSO 2 @ T6	1150	178.39
REF 3 @ T0	1010	156.67	DMSO 3 @ T0	1030	159.77
REF 3 @ T1	1090	169.08	DMSO 3 @ T1	1090	169.08
REF 3 @ T2	1080	167.53	DMSO 3 @ T2	1010	156.67
REF 3 @ T3	1140	176.84	DMSO 3 @ T3	1130	175.28
REF 3 @ T4	1030	159.77	DMSO 3 @ T4	1050	162.88
REF 3 @ T5	950	147.36	DMSO 3 @ T5	910	141.16
REF 3 @ T6	950	147.36	DMSO 3 @ T6	1050	162.88

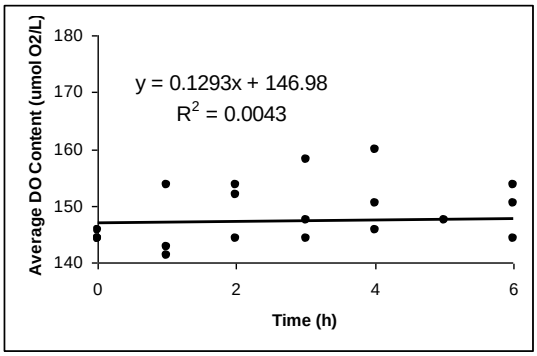




[Atrazine] = 50.0 µg/L		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
ATZ 50.0 1 @ T0	1030	159.77
ATZ 50.0 1 @ T1	1040	161.32
ATZ 50.0 1 @ T2	1080	167.53
ATZ 50.0 1 @ T3	1080	167.53
ATZ 50.0 1 @ T4	1080	167.53
ATZ 50.0 1 @ T5	960	148.91
ATZ 50.0 1 @ T6	1140	176.84
ATZ 50.0 1 @ T0	1120	173.73
ATZ 50.0 1 @ T1	1030	159.77
ATZ 50.0 1 @ T2	1030	159.77
ATZ 50.0 1 @ T3	1080	167.53
ATZ 50.0 1 @ T4	1140	176.84
ATZ 50.0 1 @ T5	910	141.16
ATZ 50.0 1 @ T6	1110	172.18
ATZ 50.0 1 @ T0	980	152.02
ATZ 50.0 1 @ T1	990	153.57
ATZ 50.0 1 @ T2	1010	156.67
ATZ 50.0 1 @ T3	1020	158.22
ATZ 50.0 1 @ T4	1030	159.77
ATZ 50.0 1 @ T5	880	136.51
ATZ 50.0 1 @ T6	1010	156.67

[Atrazine] = 500.0 µg/L		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
ATZ 500.0 1 @ T0	940	145.81
ATZ 500.0 1 @ T1	990	153.57
ATZ 500.0 1 @ T2	980	152.02
ATZ 500.0 1 @ T3	1020	158.22
ATZ 500.0 1 @ T4	1030	159.77
ATZ 500.0 1 @ T5	880	136.51
ATZ 500.0 1 @ T6	970	150.47
ATZ 500.0 1 @ T0	930	144.26
ATZ 500.0 1 @ T1	910	141.16
ATZ 500.0 1 @ T2	930	144.26
ATZ 500.0 1 @ T3	930	144.26
ATZ 500.0 1 @ T4	970	150.47
ATZ 500.0 1 @ T5	950	147.36
ATZ 500.0 1 @ T6	930	144.26
ATZ 500.0 1 @ T0	930	144.26
ATZ 500.0 1 @ T1	920	142.71
ATZ 500.0 1 @ T2	990	153.57
ATZ 500.0 1 @ T3	950	147.36
ATZ 500.0 1 @ T4	940	145.81
ATZ 500.0 1 @ T5	870	134.95
ATZ 500.0 1 @ T6	990	153.57

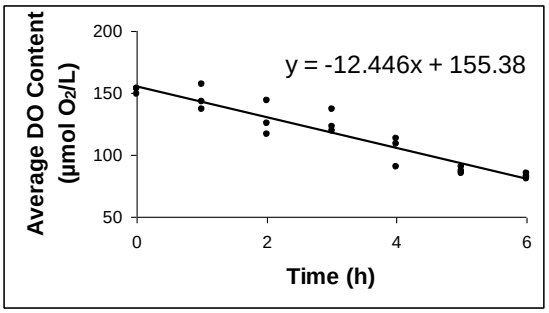


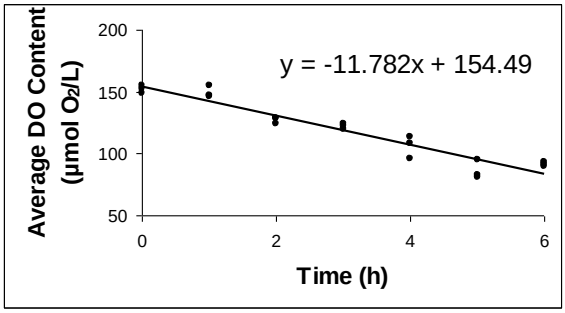


Appendix S: Raw Data for *Euglena gracilis* Respiration Experiments in the Dark with Exposure to Atrazine.

Reference		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
REF 1 @ T0	990	153.57
REF 1 @ T1	920	142.71
REF 1 @ T2	810	125.65
REF 1 @ T3	880	136.51
REF 1 @ T4	730	113.24
REF 1 @ T5	560	86.87
REF 1 @ T6	550	85.32
REF 2 @ T0	960	148.91
REF 2 @ T1	1010	156.67
REF 2 @ T2	930	144.26
REF 2 @ T3	790	122.54
REF 2 @ T4	580	89.97
REF 2 @ T5	580	89.97
REF 2 @ T6	520	80.66
REF 3 @ T0	990	153.57
REF 3 @ T1	880	136.51
REF 3 @ T2	750	116.34
REF 3 @ T3	770	119.44
REF 3 @ T4	700	108.58
REF 3 @ T5	550	85.32
REF 3 @ T6	530	82.21

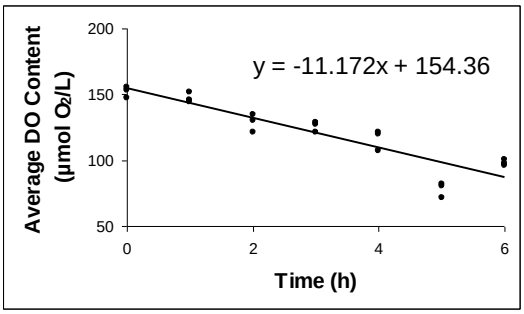
DMSO		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
DMSO 1 @ T0	1000	155.12
DMSO 1 @ T1	940	145.81
DMSO 1 @ T2	830	128.75
DMSO 1 @ T3	800	124.10
DMSO 1 @ T4	730	113.24
DMSO 1 @ T5	610	94.62
DMSO 1 @ T6	590	91.52
DMSO 2 @ T0	960	148.91
DMSO 2 @ T1	950	147.36
DMSO 2 @ T2	830	128.75
DMSO 2 @ T3	770	119.44
DMSO 2 @ T4	700	108.58
DMSO 2 @ T5	530	82.21
DMSO 2 @ T6	580	89.97
DMSO 3 @ T0	980	152.02
DMSO 3 @ T1	1000	155.12
DMSO 3 @ T2	800	124.10
DMSO 3 @ T3	790	122.54
DMSO 3 @ T4	620	96.17
DMSO 3 @ T5	520	80.66
DMSO 3 @ T6	600	93.07

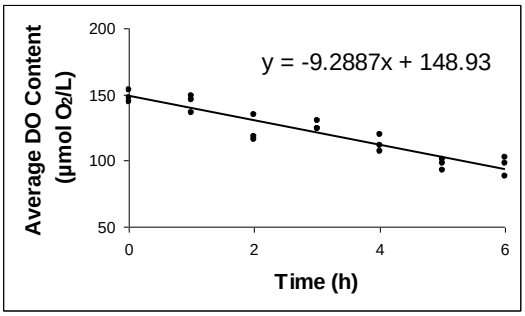




[Atrazine] = 50.0 µg/L		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
ATZ 50.0 1 @ T0	1000	155.12
ATZ 50.0 1 @ T1	930	144.26
ATZ 50.0 1 @ T2	870	134.95
ATZ 50.0 1 @ T3	780	120.99
ATZ 50.0 1 @ T4	780	120.99
ATZ 50.0 1 @ T5	530	82.21
ATZ 50.0 1 @ T6	620	96.17
ATZ 50.0 1 @ T0	990	153.57
ATZ 50.0 1 @ T1	940	145.81
ATZ 50.0 1 @ T2	780	120.99
ATZ 50.0 1 @ T3	820	127.20
ATZ 50.0 1 @ T4	770	119.44
ATZ 50.0 1 @ T5	520	80.66
ATZ 50.0 1 @ T6	650	100.83
ATZ 50.0 1 @ T0	950	147.36
ATZ 50.0 1 @ T1	980	152.02
ATZ 50.0 1 @ T2	840	130.30
ATZ 50.0 1 @ T3	830	128.75
ATZ 50.0 1 @ T4	690	107.03
ATZ 50.0 1 @ T5	460	71.35
ATZ 50.0 1 @ T6	630	97.73

[Atrazine] = 500.0 µg/L		
Vial Identifier	Volume Titrant (µL)	DO (µmol O_2/L)
ATZ 500.0 1 @ T0	950	147.36
ATZ 500.0 1 @ T1	960	148.91
ATZ 500.0 1 @ T2	870	134.95
ATZ 500.0 1 @ T3	840	130.30
ATZ 500.0 1 @ T4	690	107.03
ATZ 500.0 1 @ T5	630	97.73
ATZ 500.0 1 @ T6	570	88.42
ATZ 500.0 1 @ T0	990	153.57
ATZ 500.0 1 @ T1	940	145.81
ATZ 500.0 1 @ T2	750	116.34
ATZ 500.0 1 @ T3	800	124.10
ATZ 500.0 1 @ T4	770	119.44
ATZ 500.0 1 @ T5	650	100.83
ATZ 500.0 1 @ T6	630	97.73
ATZ 500.0 1 @ T0	930	144.26
ATZ 500.0 1 @ T1	880	136.51
ATZ 500.0 1 @ T2	760	117.89
ATZ 500.0 1 @ T3	800	124.10
ATZ 500.0 1 @ T4	720	111.69
ATZ 500.0 1 @ T5	600	93.07
ATZ 500.0 1 @ T6	660	102.38





Appendix T: Raw Data for *Euglena gracilis* Growth Inhibition Experiments with Exposure to Atrazine.

T=0	Number of Cells ($x10^4$)				
	1	2	3	Avg	Std (±)
Ref	14.4	13.0	13.2	13.5	0.78219
DMSO	16.1	15.3	15.5	15.6	0.40353
ATZ_0.5	12.9	14.6		13.8	1.21743
ATZ_5	13.9	13.9	14.3	14.0	0.22138
ATZ_50	13.1	12.4	13.7	13.1	0.68868
ATZ_500	13.5	13.1	13.2	13.3	0.18221

T=3	Number of Cells ($x10^4$)				
	1	2	3	Avg	Std (±)
Ref	13.5	14.6	14.7	14.3	0.63989
DMSO	19.0	17.5	17.1	17.9	1.02842
ATZ_0.5	20.4	20.0		20.2	0.29218
ATZ_5	19.4	17.9	18.8	18.7	0.72557
ATZ_50	16.4	17.9	16.7	17.0	0.83309
ATZ_500	18.6	16.4	15.8	16.9	1.45766

T=6	Number of Cells ($x10^4$)				
	1	2	3	Avg	Std (±)
Ref	14.3	12.4	13.6	13.4	0.97718
DMSO	17.2	17.5	17.0	17.2	0.27833
ATZ_0.5	15.7	14.8		15.3	0.63306
ATZ_5	16.2	14.8	16.3	15.7	0.83782
ATZ_50	13.7	14.3	14.5	14.2	0.39160
ATZ_500	17.6	14.4	12.9	15.0	2.38963

T=12	Number of Cells ($x10^4$)				
	1	2	3	Avg	Std (±)
Ref	18.1	18.4	20.0	18.8	1.02842
DMSO	23.6	21.5	21.2	22.1	1.30365
ATZ_0.5	21.0	20.6		20.8	0.29218
ATZ_5	23.6	20.8	18.9	21.1	2.35800
ATZ_50	20.1	18.8	18.6	19.2	0.78219
ATZ_500	24.1	20.3	19.0	21.1	2.61910

T=24	Number of Cells ($x10^4$)				
	1	2	3	Avg	Std (±)
Ref	29.9	25.5	25.0	26.8	2.65507
DMSO	27.5	23.7	26.1	25.8	1.91721
ATZ_0.5	34.1	26.7		30.4	5.25929
ATZ_5	37.2	27.8	28.1	31.0	5.32976
ATZ_50	31.3	22.1	25.8	26.4	4.64302
ATZ_500	35.3	27.4	29.4	30.7	4.11887

T=48	Number of Cells (x10^4)				
	1	2	3	Avg	Std (±)
Ref	58.7	44.9	45.1	49.6	7.91364
DMSO	50.7	46.0	49.6	48.7	2.40973
ATZ_0.5	49.5	38.1		43.8	8.08372
ATZ_5	58.6	41.5	41.9	47.3	9.78339
ATZ_50	47.6	39.4	34.3	40.4	6.70053
ATZ_500	54.2	45.1	40.7	46.7	6.85333

T=72	Number of Cells (x10^4)				
	1	2	3	Avg	Std (±)
Ref	99.6	83.5	99.4	94.2	9.24501
DMSO	76.1	72.1	95.0	81.0	12.25123
ATZ_0.5	116.3	75.9		96.1	28.53650
ATZ_5	116.1	74.4	78.5	89.7	23.01208
ATZ_50	115.5	69.2	61.3	82.0	29.31412
ATZ_500	107.9	93.6	80.5	94.0	13.70942

Appendix U: Raw Data for *Euglena gracilis* Behavioural Experiments in the Light with Exposure to Atrazine.

T=0	Number Spindle (Normal)			Number Cyst (Stressed)			Total				Percent Cyst (%)				
	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Avg	Rep1	Rep2	Rep3	Average percent cyst	Std
Reference	48	49	38	7	14	10	55	63	48	55	0.127	0.222	0.208	0.186	0.051
0.5% DMSO	66	77	90	9	11	11	75	88	101	88	0.120	0.125	0.109	0.118	0.008
50.0µg/L Atrazine	105	73	38	21	15	10	126	88	48	87	0.167	0.170	0.208	0.182	0.023
500.0µg/L Atrazine	36	36	34	7	5	4	43	41	38	41	0.163	0.122	0.105	0.130	0.030

T=3															
	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Avg	Rep1	Rep2	Rep3	Average percent cyst	Std
Reference	24	48	36	8	6	4	32	54	40	42	0.250	0.111	0.100	0.154	0.084
0.5% DMSO	22	28	31	1	4	6	23	32	37	31	0.043	0.125	0.162	0.110	0.061
50.0µg/L Atrazine	23	42	93	6	8	13	29	50	106	62	0.207	0.160	0.123	0.163	0.042
500.0µg/L Atrazine	54	22	65	23	10	29	77	32	94	68	0.299	0.313	0.309	0.307	0.007

T=6															
	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Avg	Rep1	Rep2	Rep3	Average percent cyst	Std
Reference	41	45	50	17	13	16	58	58	66	61	0.293	0.224	0.242	0.253	0.036
0.5% DMSO	60	35	31	18	12	7	78	47	38	54	0.231	0.255	0.184	0.223	0.036
50.0µg/L Atrazine	61	55	56	21	23	24	82	78	80	80	0.256	0.295	0.300	0.284	0.024
500.0µg/L Atrazine	31	36	38	20	24	34	51	60	72	61	0.392	0.400	0.472	0.421	0.044

T=24															
	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Avg	Rep1	Rep2	Rep3	Average percent cyst	Std
Reference	41	37	34	19	31	17	60	68	51	60	0.317	0.456	0.333	0.369	0.076
0.5% DMSO	63	35	53	40	19	37	103	54	90	82	0.388	0.352	0.411	0.384	0.030
50.0µg/L Atrazine	20	20	43	37	56	80	57	76	123	85	0.649	0.737	0.650	0.679	0.050
500.0µg/L Atrazine	26	30	28	62	50	39	88	80	67	78	0.705	0.625	0.582	0.637	0.062

Appendix V: Raw Data for *Euglena gracilis* Behavioural Experiments in the Dark with Exposure to Atrazine.

T=0	Number Spindle (Normal)			Number Cyst (Stressed)			Total				Percent Cyst (%)				
	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Avg	Rep1	Rep2	Rep3	Average percent cyst	Std (±)
Reference	29	13	29	3	1	2	32	14	31	26	0.094	0.071	0.065	0.077	0.015
0.5% DMSO	27	24	33	0	2	3	27	26	36	30	0.000	0.077	0.083	0.053	0.046
50.0µg/L Atrazine	56	37	42	10	3	7	66	40	49	52	0.152	0.075	0.143	0.123	0.042
500.0µg/L Atrazine	49	58	54	19	13	10	68	71	64	68	0.279	0.183	0.156	0.206	0.065

T=3															
	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Avg	Rep1	Rep2	Rep3	Average percent cyst	Std (±)
Reference	27	38	85	11	11	19	38	49	104	64	0.289	0.224	0.183	0.232	0.054
0.5% DMSO	22	37	40	12	6	6	34	43	46	41	0.353	0.140	0.130	0.208	0.126
50.0µg/L Atrazine	41	33	39	13	11	6	54	44	45	48	0.241	0.250	0.133	0.208	0.065
500.0µg/L Atrazine	43	37	31	20	8	2	63	45	33	47	0.317	0.178	0.061	0.185	0.129

T=6															
	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Avg	Rep1	Rep2	Rep3	Average percent cyst	Std (±)
Reference	31	38	34	28	24	28	59	62	62	61	0.475	0.387	0.452	0.438	0.045
0.5% DMSO	31	39	19	39	20	13	70	59	32	54	0.557	0.339	0.406	0.434	0.112
50.0µg/L Atrazine	10	21	29	10	21	29	20	42	58	40	0.500	0.500	0.500	0.500	0.000
500.0µg/L Atrazine	27	23	27	29	38	29	56	61	56	58	0.518	0.623	0.518	0.553	0.061

T=24															
	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Avg	Rep1	Rep2	Rep3	Average percent cyst	Std (±)
Reference	26	28	32	31	37	42	57	65	74	65	0.544	0.569	0.568	0.560	0.014
0.5% DMSO	16	21	32	25	50	43	41	71	75	62	0.610	0.704	0.573	0.629	0.068
50.0µg/L Atrazine	20	55	27	24	40	31	44	95	58	66	0.545	0.421	0.534	0.500	0.069
500.0µg/L Atrazine	18	25	31	46	36	16	64	61	47	57	0.719	0.590	0.340	0.550	0.192

Appendix W: Raw Data for *Anodonta grandis* Respiration Experiments with Exposure to Atrazine.

Reference

	DO Content (mmol/L) per mass (g)				
Time	**Rep#1**	**Rep#2**	**Rep#3**	**Rep#4**	**Rep#5**
1.0	7.27	5.73	6.37	5.91	5.18
2.0	7.09	5.57	6.08	5.61	5.00
3.0	6.95	5.49	5.85	5.46	4.88
4.0	6.53	5.21	5.63	5.05	4.64
5.0	6.15	4.87	5.25	4.83	4.41
6.0	5.64	4.49	4.75	4.60	3.95

0.5% DMSO

	DO Content (mmol/L) per mass (g)				
Time	**Rep#1**	**Rep#2**	**Rep#3**	**Rep#4**	**Rep#5**
1.0	6.59	4.53	6.86	7.02	6.03
2.0	6.20	4.34	6.66	7.23	5.92
3.0	6.26	4.26	6.68	6.64	5.87
4.0	5.80	3.86	6.42	6.30	5.53
5.0	5.63	3.64	5.84	5.94	4.91
6.0	5.31	3.46	5.47	5.65	4.72

0.5 µg/L Atrazine

	DO Content (mmol/L) per mass (g)				
Time	**Rep#1**	**Rep#2**	**Rep#3**	**Rep#4**	**Rep#5**
1.0	4.28	4.32	4.31	5.62	5.58
2.0	3.91	4.23	4.20	5.62	5.46
3.0	3.98	4.11	4.06	5.59	5.32
4.0	3.81	3.82	3.88	5.33	5.06
5.0	3.54	3.60	3.40	4.74	4.37
6.0	3.20	3.38	3.27	4.48	4.19

5.0 µg/L Atrazine

	DO Content (mmol/L) per mass (g)				
Time	**Rep#1**	**Rep#2**	**Rep#3**	**Rep#4**	**Rep#5**
1.0	4.78	2.78	2.51	3.28	4.73
2.0	4.58	2.68	2.38	3.19	4.53
3.0	4.50	2.59	2.33	3.14	4.37
4.0	4.30	2.48	2.25	2.95	4.13
5.0	3.99	2.30	2.12	2.77	3.84
6.0	3.83	2.17	2.04	2.65	3.81

50.0 µg/L Atrazine

	DO Content (mmol/L) per mass (g)				
Time	**Rep#1**	**Rep#2**	**Rep#3**	**Rep#4**	**Rep#5**
1.0	2.50	8.05	2.41	2.45	2.74
2.0	2.44	7.98	2.38	2.42	2.69
3.0	2.43	7.87	2.36	2.41	2.64
4.0	2.40	7.36	2.30	2.31	2.53
5.0	2.31	7.06	2.24	2.28	2.47
6.0	2.24	7.02	2.14	2.14	2.34