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The effect of β-Estradiol on freshwater algae (Pseudokirchneriella subcapitata)

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ABSTRACT

Evidence of increased presence of estrogen in aquatic environments is being found in a wide variety of organisms. Estrogens are powerful endocrine disruptors with the ability to not only mimic endogenous hormones but also alter the overall hormonal metabolism in many of these organisms. The effect of β-Estradiol on some aquatic organism is documented, however very little is known about its effect on algae. Freshwater algae (*Pseudokirchneriella subcapitata*) were used in a growth inhibition tests, examining the effects of β-Estradiol on algae population growth. A series of 72 hour growth inhibition tests were performed using replicates of ten concentrations of β-Estradiol, a negative control and a positive control (NaCl). The population size of the algae was estimated by measuring absorbance. A single factor ANOVA analysis of the results indicated that there was no effect of β-Estradiol on the algae population size. This suggests that accumulation of β-Estradiol is occurring within the algae and the potential for bioaccumulation within the algae natural aquatic ecosystem.

INTRODUCTION

Bioaccumulation

Bioaccumulation is the accumulation of a specific compound within an organism (Brady and Weil 2010) which has the ability to affect all living organisms especially when it involves harmful chemicals (Chojnacka and Mikulewicz 2014). Bioaccumulation is the outcome of dynamic equilibrium between the uptake of a compound and the elimination of that compound and can vary in degrees, in regards to the toxic effects that it has on the organism. This is based on the amount of the compound that has accumulated in the organism. Many factors are involved when bioaccumulation takes place such as the amount of the compound including the concentration, as well as the type and size of the organism that is being affected. A proper understanding of bioaccumulation can be used to assess the risk of the chemicals found in the environment and to determine an effective way to manage the use and emission of the chemical (Chojnacka and Mikulewicz 2014).

The introduction of an invasive species, poor water quality, climate change and any other environmental changes can increase the likelihood of bioaccumulation of a persistent chemical taking place. In order to prevent distribution of contaminants the ability to anticipate outcomes of complex ecological interactions within an ecosystem is essential. This is especially true in an aquatic ecosystem where there is a greater risk of harmful and persistent chemicals entering the ecosystem. The presence of water and the physiology of organisms found in aquatic environments is one of the reason bioaccumulation can occur more readily. Fish have gills which are permeable wet membranes this allow chemicals to very easily enter the body of the fish. During the consumption of food by an aquatic organism there is an increase chance that the

chemical will enter their body because most often when they open their mouth to take in food they also get water which doubles the chance of the chemical entering the body of the organism and thus bioaccumulation may then occur (Ng and Gray 2009). A food web is a model used to display and interpret the patterns of how the trophic levels are connected within an ecosystem in which there is a movement of biomass and energy. As mentioned previously, chemicals are taken up by the organism in a number of ways, once the compounds has entered the body it has the ability to be stored in the fat tissue of the organism and accumulate. When the concentration of the toxin is higher in the aquatic organism then the concentration found in the environment around it bioaccumulation is taking place (Taffi, Paoletti, Liò *et al.* 2014).

β-Estradiol

β-Estradiol is a human sex hormone in the steroid category and is the primary sex hormone in females, it plays an essential role in both the female mammalian estrous and menstrual reproductive cycles. In order for the proper development and maintenance of the tissue in the reproductive system of the mammalian females β-Estradiol is crucial (Ryan 1982). Not only that but it also has important effects on many other tissues including bone (Ryan 1982), breasts, skin and brain (Ying, Kookana, and Ru 2002).

There have been a large number of studies in the last decade that have assessed the anthropogenic demand on the environment. As mentioned earlier, recently there has been growing concern about pharmaceutical products entering ecosystems after human consumption (through feces and urine). There are such a large number of pharmaceuticals consumed that analgesics, antibiotics and contraceptives can all be found in almost every aquatic ecosystem.

One of the main focuses when looking at pharmaceutical products found in aquatic environment is on contraceptives drugs. This is because they are powerful endocrine disruptors (EDs) which have the ability to not only mimic endogenous hormones but also alter the overall hormonal metabolism in a large number of organisms. Endocrine disruptors include compounds such as phytoestrogens and many synthetic chemicals (Souza, Hallgren, Balseiro *et al.* 2013).

The increased presence of estrogen in aquatic environments can now be seen in a wide variety of organisms. This is especially true of water that is located downstream from effluent outlets. There are many ways for aquatic organism to take up the estrogen that is found in the water. All of which can cause issues such as biomagnifcation and bioaccumulation. This is why studies looking into the effects that the estrogen has on the organisms are so critical (Lai, Scrimshaw and Lester 2002). It has been determined that in the environment the distribution of estrogens is affected by their physicochemical properties as well as the specific environmental conditions of the area. Estrogens undergo a number of different transformations in the liver. So by the time estrogens are excreted they have usually been transformed into inactive compounds of sulphuric and glucuronic acid. These compounds can then be returned to free steroids by bacteria that are found in the environment even though they do not directly possess biological activity. Microorganisms that can be found in raw sewage and sewage treatment plants are able to alter inactive estrogens into active estrogens which are then released into the environment. Studies have estimated that estrogens that are present in water and sediment have a half-life of approximately 2-6 days (Ying, Kookana, and Ru 2002).

The reproductive systems of aquatic organisms are being affected by the presence of estrogens in their environment, for example the feminization of male fish that have been exposed to sewage treatment plant effluents. This is caused by endocrine disrupters. There are several

drugs that can influence the endocrine system. One of the most well-known examples of this would be birth control pills. Many of the pharmaceuticals and natural hormones are excreted by humans and end up in municipal sewage, they are not removed at sewage treatment plants and so they enter waterways because little is known about how to properly deal with natural and synthetic estrogens and its behaviour when present in wastewater (Ternes, Stumpf, Mueller *et al.* 1999).

Pharmaceuticals are designed to be effective at very low concentrations; however they also have the ability to be harmful to both those who they are meant for and those who take them in unintentionally. They are designed for humans and other animals but often have side effects. It has been reported that phyto-plankton, zoo-plankton and other aquatic organisms have and can be affected by a mixture of pharmaceuticals. In some aquatic organism these mixtures are known to inhibit cell proliferation (Vannini, Domingo and Marsoni *et al.* 2011).

Pseudokirchneriella subcapitata (Freshwater algae)

Algae have a large role in aquatic ecosystems and they are also known for being relatively sensitive to toxins in general (Moreira-Santos, Soares, Ribeiro 2003). Despite the fact that *Pseudokirchneriella subcapitata* is the most commonly used species of algae for toxicity testing there is not an abundance of information available on the species. *P. subcapitata* can be found living in freshwater ponds, lakes and rivers and are known as a planktonic species. The algal cells are a helical shape, which is most often has a semi-circularly curved when in the vegetative phase. They have a diameter of 154 - 360°, an arc range of 4.8 to 10.8 μm, a width of 1.6 - 4.4 μm and depth/width ratio of 1.7 - 4.1. Reproduction occurs when the mother cell divides into 2, 4, or 8 autospores (Aruoja 2011). *Pseudokirchneriella subcapitata* formerly *Selenastrum*

capricornutum (Chlorophyceae) is found in most North American fresh waters (Katsumata, Koike, Nishikawa *et al.,* 2006, Cáceres, Megharaj, Naidu 2008).

Since *P. subcapitata* is known for its sensitivity to the presence of toxic substances it becomes an ideal choice for it to be used as a bio-indicator species to assess the levels of nutrients or toxic substances in freshwater environments. It is very commonly used in the area of ecotoxicology. With more than ten thousand chemicals that are currently used in agricultural and industry applications there are many toxins possibly getting into the environment. Many of the chemicals used have unpredictable effects when discharged into aquatic ecosystems. Because of this there is a very large interest in estimating the possible effects before contamination occurs (Katsumata, Koike, Nishikawa *et al.,* 2006).

Microalgae are essential in many aquatic ecosystems, they are primary producers, and can be a valuable food source for other organisms. They also help with the production of dissolved oxygen as well as balance the amount of oxygen found, this is just one of the many ways that algae helps create optimal conditions in the aquatic ecosystems. Some microalgae species are known to be extremely sensitive when contamination is present in their environment. Many of those same sensitive species are known as and often referred to as indictors of contamination (Lai, Hou, *et al.* 2009). However some other species of algae have an ability to tolerate many of these harmful compounds that are now present in their environment. For example studies have shown that *Tetraselmis chuii* is able to tolerate both copper and lead (Debelius, Forja, *et al.* 2009).

Pseudokirchneriella subcapitata is a unicellular algae species that is non-motile and crescent shaped. This species is the most commonly used algae for toxicology testing because of

its known widespread sensitivity to toxicants (Aruoja, Moosus, Kahru *et al.,* 2014, Cáceres, Megharaj, Naidu 2008). Algae are known for being one of the most essential organisms found in aquatic ecosystems. They are able to provide both oxygen and are a very important part of the food chain with the algae itself being a food source for higher trophic organisms in their ecosystem. Thus, if a toxin or chemical affects the algae it can in turn affect organisms that depend on the algae, such as fish and invertebrates. This could result in problems such as oxygen depletion, decreased primary productivity, and affect the overall food chain of the aquatic ecosystem (Satyavani, Chandrasehar *et al.,* 2012).

There is a need for research on the effect of β-Estradiol on freshwater algae (*Pseudokirchneriella subcapitata*). This becomes evident due to the lack of information available on this topic. More research on the effects that β-Estradiol cause is essential, estrogens have a strong potential to cause serious problem for the ecosystems and the species that live within it since they are endocrine disrupters. This gives them the ability to make dramatic changes to a species such as full or partial feminization. The present of estrogens in the waters is becoming an increased problem due to population growth and lack of treatments to properly remove them from the sewage water before they are released. Based on the negative effect that β-Estradiol has on many aquatic organisms higher on the food chain it is highly likely that β-Estradiol will also have an effect on algae.

The use of a 72 hour growth inhibition test with *Pseudokirchneriella subcapitata* (freshwater algae) can be a very effective technique to show some of the effects β-Estradiol can cause. This is because this species of algae is the most commonly used for toxicity testing. Furthermore it is also very likely that this species of algae to be found naturally in areas that are effected by the presences of β-Estradiol. Thus not only providing data that can be used to

understand how β-Estradiol can affect general aquatic fauna and flora but specifically *Pseudokirchneriella subcapitata* as well.

METHODS

The research that I have conducted looked at the effect of β-Estradiol on the freshwater algae *Pseudokirchneriella subcapitata*. This species of algae was selected because that is the same species used in the Environment Canada protocol (March 2007) and that protocol was followed, with a few minor changes. A different growth medium was used, based on recommendations made for the toxicology lab which looked at the effect of effluents of freshwater algae using growth inhibition tests, the growth medium that was used was BBM. I have maintained the algae culture since the culture was obtained for environmental toxicology (ENSC 350) in the winter term of 2014.

Determination of β-Estradiol concentrations

A review of literature was done to determine the levels of β-Estradiol that are found in natural aquatic ecosystems and the paper entitled "Levels of six estrogens in water and sediment from three rivers in Tianjin area, China" by Lei, Huang, Zhou, Wang, and Wang (2009) was selected as a reference for the β-Estradiol that was used in the testing process. It provided a number of different β-Estradiol concentrations detected in water environments all over the world. The concentrations provided in the paper were examined and average concentration was determined. The average was used as the baseline concentration of β-Estradiol. That number was determined to be 8.277×10^{-9} mg/mL. Due to the fact that measuring that exact amount of β-Estradiol would be very difficult the number was slightly adjusted when the solutions were prepared. Once it the lab the baseline concentration changed from 8.277×10^{-9} mg/mL to 8.440

x 10^{-9} mg/mL. From there five concentrations above (2.70 x 10^{-7} mg/ mL, 1.35 x 10^{-7} mg/mL, 6.75 x 10^{-8} mg/mL, 3.38 x 10^{-8} mg/mL, and 1.96 x 10^{-8} mg/mL) and four concentrations below (4.22 x 10^{-9} mg/mL, 2.11 x 10^{-9} mg/mL, 1.05 x 10^{-9} mg/mL and 5.27 x 10^{-10} mg/mL) the baseline were selected as the ten concentrations of β-Estradiol that were tested. All the concentrations differ from by a factor of two; so that all the β-Estradiol concentrations differ by an equal factor. Two preliminary growth inhibition tests were be performed with the ten selected concentrations of β-Estradiol.

Preliminary Testing

After the two preliminary growth inhibition tests were set up a preliminary test was performed to determine the wavelength of maximum absorbance. This was done in order to determine the wavelength to set the micro-plate reader to; the reader has a total of 5 wavelength settings to choose from. A spectrophotometer was used to determine the wavelength with the highest absorbance reading for the solution. The solution consisted of distilled water and algae. The solution was tested at the 5 wavelength settings that are featured on the micro-plate reader (340nm, 415nm, 450nm, 595nm, 655nm). Wavelength of maximum absorbance was 340nm. This was the wavelength setting sectioned to be used when all of the plates were read by the micro-plate reader.

Testing Periods

After the wavelength and β-Estradiol concentrations were determined the testing period began. My project included four periods of testing. Each testing period included 10, 72 Growth Inhibition Tests. All 10 plates were set up on one day. After the 3 day growth period each of the plates were examined using a micro-plate reader. The micro-plate reader provided the

absorbance reading for each of the wells in the plate. The absorbance is an indication of cell biomass. The cell biomass indicates the amount of algal cells present. This can be used to determine if the β-Estradiol has an effect on algal population growth. This testing process was replied during separate weeks. A total of 40 tests were performed, generating 200 replicates for each concentration of β-Estradiol. A negative control (distilled water) and a positive control of NaCl (0.87 g/L) were also tested, generating 200 replicates for each. Sodium chloride was used at this concentration as indicated by Santos, Vicensotti and Monteiro (2007) to have an effect on *Pseudokirchneriella subcapitata* and can be used as a reference toxicant. After the testing was completed the absorbance values obtained from the plate reader will be analyzed and determine to be statically significant or not based on ANOVA analysis followed by a regression.

96 well mirocplate used. The outside wells of the plate were filled with sterile deionized water (in order to keep the humidity high in the remaining wells). The BBM enrichment medium concentration that was used was 100%. There were 5 replicates of each β-Estradiol concentration as well as 5 replicates of both the negative and positive control in the well plate. In each well there was 10μL of 100% BBM (concentration that the best algal growth occurs at), 10μL of the algal inoculum (which was made with $NaHCO_3$, *Pseudokirchneriella subcapitata* and provided 2200 cells per well), and 200μL of β-Estradiol at various concentrations, negative control or positive control were added. The only wells that did not receive a treatment, negative control or positive control were the outside wells.

The well plates were stored in the growth chamber for 72 hours; for a 72 hour inhibition test. The setting for the controlled environmental conditions included a LD 12:12, at 24^{o}C, cool white bulbs, 2000-4000 lux, and gentle agitation on a shaker (60 RPM). The concentration of

cells in the original mixture was used to determine the cell count. All of the processes were based on the Environmental Canada protocol.

RESULTS

Figure 1 (see below) illustrates the relation between the ten β-Estradiol concentrations (log scale, base 10) and the average absorbance after the 72 hour growth inhibition test. This graph does not include the controls. The data used was 50 replicates of each of the ten β-Estradiol concentrations, negative control and positive control which were obtained from the fourth and final testing period. A one factor ANOVA analysis was conducted to compare the effect of the treatments on algae population growth. There was no difference in effect of treatments on the algae at the $p \leq 0.05$ level for the ten concentrations and two controls [$F(11, 588) = 1.741685$, $p = 0.061153$].

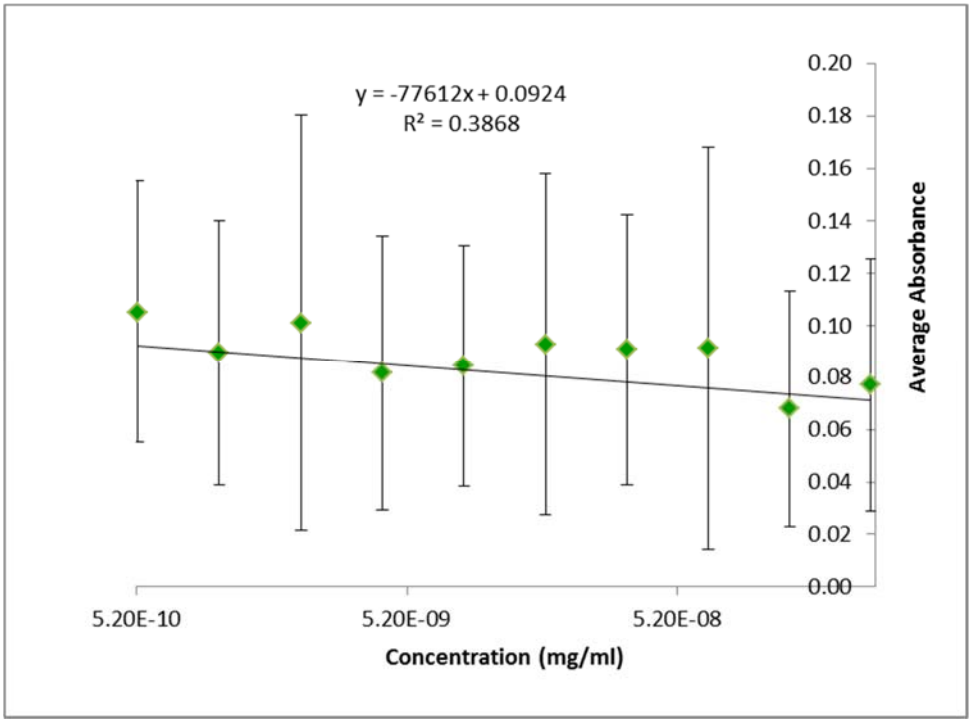

Figure 1. The relationship between ten β-Estradiol concentrations (a logarithmic scale with base 10) and the average absorbance after 72 hour growth inhibition test.

DISCUSSION

The aim of this research was to examine the effect of β-Estradiol on fresh water algae (*Pseudokirchneriella subcapitata).* Ten different concentrations of β-Estradiol were used for growth inhibition tests as well as a negative control and a positive control (NaCl). There was a total of four testing periods completed. Ten growth inhibition tests were performed in each of the four testing periods. After the first three testing periods data analysis was performed, based on the graph it was clear that three of the concentrations have absorbance averages indication algal cell biomass that did not fit in with the rest of the data. This could be due to one of two reasons: micro-plate reader error or error to due to lack of randomization. An additional test was conducted with those three concentrations to see if the micro-plate reader was the cause of the error. This test was not conclusive. This led to the decision to perform one final testing period (this was testing period number four), this time with randomization. The results that were obtained from this testing period were analyzed as used as the results for this paper. The data used was 50 replicates of each of the ten β-Estradiol concentrations, negative control and positive control.

According to the one factor ANOVA analysis there is no difference in effect that β-Estradiol concentration and the controls had on the freshwater algae. There are a number of conclusions that can be drawn from this information. One conclusion is that this species of fresh water algae (*Pseudokirchneriella subcapitata)* is not a good indicator species for this particular form of estrogen. As noted previously this species of algae is very commonly used when preforming toxicological tests because they are known to have a widespread sensitivity to toxicants (Aruoja, Moosus, Kahru *et al.,* 2014, Cáceres, Megharaj, Naidu 2008). Earlier studies have determined that *P. subcapitata* is a good indicator species when nutrients and toxic

compounds are present; both of these are commonly found in freshwater environments (Katsumata, Koike, Nishikawa *et al.,* 2006). Other studies have shown that *P. subcapitata* and other algae species are able to tolerate compounds such as copper, lead (Debelius, Forja, *et al.* 2009) and some pharmaceuticals (Vannini, Domingo and Marsoni *et al.* 2011).

Another observation that can be made is that *P. subcapitata* may have the ability to accumulate and tolerate the presence of compounds such as β-Estradiol, specifically at low concentration such as the ones that were tested. Studies that shown that *P. subcapitata* has the ability to not only tolerate but to have continued cell proliferation with the presences of compounds such as Atenolol, Bezafibrate, Carbamazepine, Ciprofloxacin, Lincomycin, Ofloxacin, Sulfamethoxazole, Cyclophosphamide, Ibuprofen, Ranitidine, and Salbutamol. All of these are known as pharmaceutically-active compounds (Vannini, Domingo and Marsoni *et al.* 2011).

P. subcapitata ability to tolerance and accumulate such compounds led to the last observation which is the potential for bioaccumulation. This experiment showed that the different concentrations of β-Estradiol did not have different effects on the algae population growth, which means there is a possibility that the algae is able to survive even with the presence of β-Estradiol. Bioaccumulation has potential to be extremely harmful especially in aquatic ecosystem much like the habitats that *P. subcapitata* are naturally found in (Ng and Gray 2009). Freshwater algae such as *P. subcapitata* are primary producers and a very important source of food for other organisms (Lai, Hou *et al.* 2009). *P. subcapitata* is at the bottom of a very complex and large food chain in the aquatic ecosystems in which they are found. This means that if compounds are accumulating in the algae it has the potential to bio-accumulate in a number of organisms that are higher on the food chain such as fish which studies have already shown to be

negatively affected by β-Estradiol often leading to feminization of the male fish. The β-Estradiol causes feminization because it is an endocrine disruptor; this when leads to problems with reproduction within the fish populations (Park, Aoki, Lee *et al.* 2010). This research supports the thought that bioaccumulation in these aquatic environments has the potential to start at the level of the fresh water algae because the presence of β-Estradiol at these levels does not cause complete death of the algae. The accumulation of β-Estradiol in *P. subcapitata* can cause a number of serious problems for all organisms that are presence in the aquatic ecosystem that the algae are found in, with the major concern being bioaccumulation. Continued research looking into the effects β-Estradiol on freshwater algae will provide more evidence. Suggestions for future research include looking at more concentrations of β-Estradiol and further analysis on the algae cell tissue to see if there is a more cellular level effect of the β-Estradiol.

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