The Effects of Hydroxypropyl-β-Cyclodextrin on the American Flagfish (*Jordanella floridae*) Over One Complete Life-Cycle

by

Jordan Christopher Anderson

A Thesis Submitted in Partial Fulfillment

of the Requirements of the Degree of

Masters of Science

in

The Faculty of Science

Applied Bioscience

University of Ontario Institute of Technology

August, 2013

Abstract

Understanding the impacts of pharmaceuticals and personal care products (PPCPs) on aquatic ecosystems is an important issue in aquatic toxicology. Many PPCPs have been shown to cause effects on aquatic biota within detected environmental ranges. One compound of particular interest is hydroxylpropyl-β-cyclodextrin (HPβCD), the active ingredient in Febreeze®, and widely used for many applications. HPβCD is amphiphilic, toroidal in shape, and able to form non-covalent inclusion complexes with a variety of guest molecules. HPβCD has been shown to reduce volatility as well as improve the aqueous solubility of apolar guest compounds. As such, the use of HPβCD in the pharmaceutical and personal care industry has dramatically increased. With increasing potential for entering the environment through wastewater treatment plant (WWTP) effluent, HPβCD poses an unknown risk to non-target aquatic biota. As a result, a 145-day chronic full life-cycle exposure using American flagfish (*Jordanella floridae*) was completed using flow-through concentrations of 0 (control), 5, 16, 50,160, 500, and 1600 μg/L of HPβCD maintained via a peristaltic pump. No significant differences were observed in growth, condition factor (K) and hepatosomatic index (HSI) when chronically exposed to HPβCD ($P \leq 0.05$). A significant increase in female gonadosomatic index (GSI) occurred in those exposed to HPβCD ($P \leq 0.05$). A reduction in the time to reach steady-state spawning occurred at 1600 μg/L of HPβCD, while an increase in the total number of days eggs were laid was observed at 16, 160, and 1600 μg/L of HPβCD ($P \leq 0.05$). A temporary reduction in offspring total length occurred at 21 days post hatch at 5, 16, 50, 160, and 500 μg/L of HPβCD ($P \leq 0.05$). Finally, larval offspring from parents exposed to HPβCD showed a moderate 3-fold decrease in tolerance to acute copper toxicity.

Keywords: Hydroxypropyl-β-Cyclodextin, PPCP, Chronic, American Flagfish (*Jordanella floridae*), Reproduction

Acknowledgements

I would like to thank everyone who has helped me complete this thesis over the past two years:

Dr. Douglas Holdway, my supervisor, for not only his incredible knowledge in the field of aquatic toxicology, but his never-ending support and guidance throughout the completion of my project.

My committee members, Dr. Andrea Kirkwood and Dr. Jean-Paul Desaulniers, for their willingness to listen to my ideas, aid in the designing of my experiments, and interpretation of my results.

John Guchardi, for his help in design, setup, and execution of all my experiments and for his unmatched patience and technical knowhow.

Lindsay Beyger, for her willingness to take the time to pass on her knowledge of maintaining and breeding flagfish. Also, for being a great friend and lab mate.

Zacharias Pandelides and lab mates for their continual help and friendship.

My friends, family, and Rachel for their unconditional support and encouragement.

Table of Contents

Index to Figures

Index to Tables

Index to Appendices

List of Abbreviations

ADME - Absorption, distribution, metabolism, and elimination

ANOVA - Analysis of variance

d - Day

DPH - Days post hatch

DDT - Dichlorodiphenyltrichloroethane

DDE - Dichlorodiphenyldichloroethylene

EC_{50} - Effective concentration that causes 50% response in test organisms

EE2 - 17α-ethynylestradiol

EROD - Ethoxyresorufin-O-deethylase

GC – Gas Chromatography

GSI - Gonadosomatic index

h - Hour

HPβCD - Hydroxypropyl-β-cyclodextrin

HPLC - High performance liquid chromatography

HSI - Hepatosomatic index

K - Condition Factor

K_{OC} – Absorption coefficient

K_{OW} - Octanol-water coefficient

LC_{50} - Lethal concentration that causes 50% mortality in test organisms

n - Number of subjects

MS-222 - Tricaine methanesulfonate

PPCPs - Pharmaceutical and personal care products

SE - Standard error

WWTP - Wastewater treatment plant

1.0 Literature Review

1.1 Introduction

In recent years, there has been heightened environmental awareness from both environmental scientists and public stakeholders regarding the increased use and disposal of pharmaceuticals and personal care products (PPCPs). Pharmaceuticals are a highly diverse group of medicinal compounds used to diagnose and treat disease in biological systems, most commonly humans (Corcoran *et al*., 2010). Personal care products are used daily by humans for hygienic, cleaning, and deodorizing reasons, and include, but are not limited to, shampoos, conditioners, fabric softeners, detergents, deodorizers, and other cleaning agents. Recently, the consumption of PPCPs has increased dramatically due to their ease of use, effectiveness, and high marketability. One group of emerging PPCPs are known as cyclodextrins which are used in both pharmaceutical and personal care industries (Del Valle, 2004). Cyclodextrins have become extremely sought after compounds due to their diverse uses in the textile industry, odour elimination, and within drug delivery systems (Del Valle, 2004). The high use of PPCPs and nature of their disposal often result in the deposition of PPCPs into the aquatic environment. In particular, pharmaceuticals are often biologically active and therefore may pose a risk to non-target organisms (Cleuvers, 2004). As a result, their impact on non-target biota should be investigated in chronically Examining the impacts over a complete life-cycle of a model test species allows for an in depth analysis of, growth, reproduction, and survivorship.

1.2 Pharmaceuticals and Personal Care Products in the Environment

Currently, pharmaceuticals represent nearly 4000 different compounds, a number that increases by nearly 7% annually (Monteiro and Boxall, 2010; Corcoran *et al*., 2010; IMS Health, 2012). In 2011, the pharmaceutical industry had a global market value of over $950 billion US dollars (IMS Health, 2012). Due to the broadness of its definition, personal care product consumption is much more difficult to estimate; however, over 1000 unique compounds are thought to be currently in use (Daughton and Ternes, 1999). This high rate of PPCP consumption has raised environmental concerns as of late. Detection of these compounds in the environment has occurred since the 1970s; it was not until the late 1990s that the effects on non-target organisms were first analyzed (Marsalek, 2008). Additionally, the advancement of analytical techniques has allowed the environmental detection of numerous xenobiotic PPCPs in recent years (Marsalek, 2008). Many PPCPs, particularly pharmaceuticals are biologically active and often elicit non-target effects on wildlife (Cleuvers, 2004).

Based on the nature of consumption and disposal, PPCPs most often impact local watersheds and most commonly enter the environment through wastewater discharge (Yang and Metcalfe, 2006; Corcoran *et al*., 2010). Pharmaceuticals typically enter wastewater systems though fecal and urine elimination as well as discarded medication (Rosi-Marshall and Royer, 2012). Due to their mode of use, personal care products are most often discharged into local sewage systems after use. PPCPs can be found as either the parental compound or as degradation products, both of which may pose a threat to ecosystem health.

PPCPs in wastewater enter the environment via direct release, septic bed leaching, and most commonly, through wastewater treatment plants (WWTP) (Snyder *et al.,* 2003). Septic beds and direct release methods of dealing with wastewater are more common in rural regions in North America, with a few exceptions. It is estimated that roughly one million homes in the United States have limited sewage treatment, while a number of Canadian cities do not treat sewage at all (Daughton and Ternes, 1999). Also, it has been reported that nearly one trillion liters of untreated wastewater are released into the environment each year (Daughton and Ternes, 1999). WWTPs are common in highly populated and developed regions. As a result, WWTPs often service a high number of people and therefore produce a large volume of biosolids and effluent. Biosolids are often used to supplement nutrients in agricultural settings, while effluent is generally released into local water bodies after treatment (Yang and Metcalfe, 2006; Corcoran *et al*., 2010).

The major focus of most WWTPs is the removal of organic matter, and as a result, many PPCPs pass through with very little degradation (Snyder *et al.,* 2003). This means that PPCPs in the environment range from nanogram to microgram per liter (ppt to ppb range) (Rosi-Marshall and Royer, 2012). Moreover, the constant release of effluent into the environment has created a pseudo-persistence of PPCPs, allowing the maintenance of xenobiotic concentrations within local ecosystems even when the compound is not inherently persistent (Durán-Alvarez *et al.,* 2009).

Multiple studies have shown that chronic exposure to trace levels of PPCPs can elicit negative biological effects on aquatic organisms. For example, Kidd *et al.*(2007) published a seven year whole-lake study on the chronic effects of 17α-ethynylestradiol (EE2) on the fathead minnow. EE2 is commonly used as a synthetic estrogen in

contraceptives, and the results of this study showed that a concentration of 5-6 ng/L EE2 caused the entire fathead minnow population to collapse. The collapse was attributed to the feminization of males, intersex gonadal development and the production of vitellogenin, a yolk precursor protein in males. These results indicated that even at trace levels, PPCPs may cause significant effects on wild fish populations (Kidd *et al.,* 2007).

The fate of PPCPs in the aquatic environment is often dependant on three factors. The first factor is a daily or seasonal pattern of use; most PPCPs are used on a daily basis with a few exceptions (Monteiro and Boxall, 2010). The second factor is the prevailing environmental conditions of the receiving waters. Stagnant receiving water often has higher xenobiotic concentrations, while flowing water tends to reduce concentrations of contaminants (Monteiro and Boxall, 2010). The receiving water chemistry also plays a major role in PPCP fate (Monteiro and Boxall, 2010). Of particular interest is pH, as it can result in the alteration of chemical species as well as the preservation or break down of certain PPCPs. The final factor considered when determining PPCP fate in the environment are the chemical properties of the compounds. Solubility levels and octanol-water coefficients (K_{ow}) are often viewed as important factors in determining environmental fate (Meylan *et al., 1998;* Monteiro and Boxall, 2010). Solubility indicates how well a compound is able to dissolve in water, therefore indicating the environmental compartment it is most likely to partition in. K_{ow} is used to indicate a compounds preference for a lipid or water based environment (Meylan *et al.,* 1998). This not only provides information on where a particular PPCP is located in an environment, but also where it may be found within an organism that has consumed or absorbed it. A compound with a log K_{ow} greater than one tends not to be excreted through urine or feces,

but rather collect within the high lipid content tissues of aquatic organisms (Meylan *et al.*, 1998). This will ultimately pose a risk for bioaccumulation in organisms at higher trophic levels, including humans, exposing such organisms to concentrations greater than those found within the environment (Meylan *et al.*, 1998). On the other hand, compounds which are more water soluble and have a log K_{ow} less than one, are often absorbed and excreted more readily (Meylan *et al.*, 1998). To understand the ability for PPCPs to enter the environment, the processes of the main point source, WWTPs, should be examined.

1.3 Wastewater Treatment Plants

1.3.1 Processing Wastewater

Wastewater treatment plants are known as one of the largest point source polluters of PPCPs. This is generally attributed to the large number of people they service, particularly in urbanized areas. Furthermore, WWTPs were not designed to remove PPCPs, but rather, focus on the removal of nutrient matter, including nitrogen and phosphorus (Pasquini *et al.*, 2013). To understand the fate of PPCPs within WWTPs, the process of treating wastewater must be understood. There are generally five stages of wastewater treatment; pretreatment, primary treatment, secondary treatment, tertiary treatment and sludge treatment (Batt *et al.*, 2007). Each stage has a defined role in treating the wastewater.

Pretreatment is the first step of the wastewater treatment process and involves the physical removal of large debris. The influent water passes through large screens, removing garbage which may otherwise interfere with treatment in the subsequent steps

(Carballa *et al.,* 2004). This water, when free of large debris, is sent to the primary treatment stage.

Primary treatment, also known as the clarifying stage, removes any remaining solid waste that is present in the wastewater (Batt *et al.,* 2007). This is done through a settling and removal process driven by the differential density of the solid waste. The remaining water is sent to the secondary treatment stage while the solid waste is collected for sludge treatment. This process is very similar in nearly all WWTPs.

Secondary treatment involves biological processes used to remove nutrients, including, PO_4, NO3, NH_3, and NH_4 from the influent water. Although this stage can vary widely, the general process and goals are the same. The influent water enters a biological reactor where the target nutrients, phosphorus, and nitrogen in particular, are consumed and removed by microbes (Carballa *et al.,* 2004). WWTPs generally have an aerobic or anaerobic biological reactor, while many have both; the type of bioreactor is dependent on the WWTP. Treated water from the biological reactor is then collected into a clarifier, similar to the primary treatment, where excess sludge and bacteria from the reactor can settle out (Carballa *et al.,* 2004). This sludge is collected and combined with waste from the primary treatment. Depending on location and the level of sophistication of the WWTP, the remaining water is either released as effluent into a local water body or it is sent to tertiary treatment at this stage.

Sludge that is collected from the primary and secondary clarifiers is often used in the agricultural industry as an effective fertilizer (Yang and Metcalfe, 2006; Corcoran *et al*., 2010). However, due to the presence of pollutants and pathogens present, it must be

treated prior to use. The addition of microbial populations causes the sludge to become 'activated', resulting in the reduction of some harmful pollutants and human pathogens (Carballa *et al.,* 2004). Based on treatment efficiency, the end product can then be approved for agricultural use. However, many PPCPs can still be present within the treated sludge (Yang and Metcalfe, 2006).

Tertiary treatment is most often used as a disinfection stage, removing any pathogens or other biological entities, including bacteria. Chlorination and ozonation are the two most widely used methods for tertiary treatment. After treatment, the water is released as effluent into local water bodies. This effluent is void of solid waste, many nutrients and most pathogens (Carballa *et al.,* 2004).

1.3.2 Fate of PPCPs in WWTPs

Due to their vast diversity and difficulty to remove, there is usually no dedicated step for the elimination of PPCPs in WWTPs. However indirect removal does occur for some PPCPs (Carballa *et al.,* 2004). A major factor in PPCP removal is the adsorption coefficient (K_{oc}), a measurement that determines how readily a compound adsorbs to a surface. The higher the adsorption coefficient, the more likely a compound is to adsorb to solid particles within the sludge such as clay, sediments, and microorganisms within the WWTP (Carballa *et al.,* 2004; Ratola *et al.,* 2012). By not adhering to solid particles during this stage, PPCPs will remain in the aqueous phase, thus increasing the risk of deposition in receiving aquatic environments (Carballa *et al.,* 2004). The adsorbed compounds may still enter the environment through the use of sludge in agricultural practices (Yang and Metcalfe, 2006; Corcoran *et al*., 2010). In addition to the adsorption coefficient, K_{ow} is also a good indicator of the fate of PPCPs in WWTPs. A log K_{ow}

above one will result in a greater likelihood that the compound will be removed with lipids during sludge removal. On the other hand, a compound with a log K_{ow} below one will most often end up in the aqueous phase of the WWTP increasing the probability of the PPCP entering the aquatic environment (Kanda *et al.*, 2003).

Microbiota metabolism may contribute to the removal of PPCPs from wastewater. Many bacteria are able to metabolize the PPCPs that enter the biological reactors of the WWTP. However, this is not always beneficial; many PPCPs and other pollutants have metabolites that are as toxic, or in some cases more toxic than their parent compound. A well-documented example of this is dichlorodiphenyltrichloroethane, commonly known as DDT, with its conversion to dichlorodiphenyldichloroethylene (DDE) (Kitamura *et al.*, 2001). DDE has been known to be highly toxic to many non-target organisms, specifically birds of prey.

The rate of consumption as well as the rate of development of new PPCPs appears to be growing each year. As a result, the determination of environmental fate is often understudied or difficult to determine. Cyclodextrins are prime examples of both emerging PPCPs and environmental contaminants.

1.4 Cyclodextrins

Cyclodextrins are cyclic oligiosaccharides consisting of 6, 7, or 8 glucopyranose units linked by α-(1,4) bonds. Cyclodextrins are most commonly present as either α, β, or γ containing 6, 7, or 8 glucopyranose subunits, respectively (Figure 1) (Del Valle, 2004). The resulting toroidal configuration of the cyclodextrin molecule allows for a highly useful binding cavity; see Figure 2 (Szejtli, 1998).

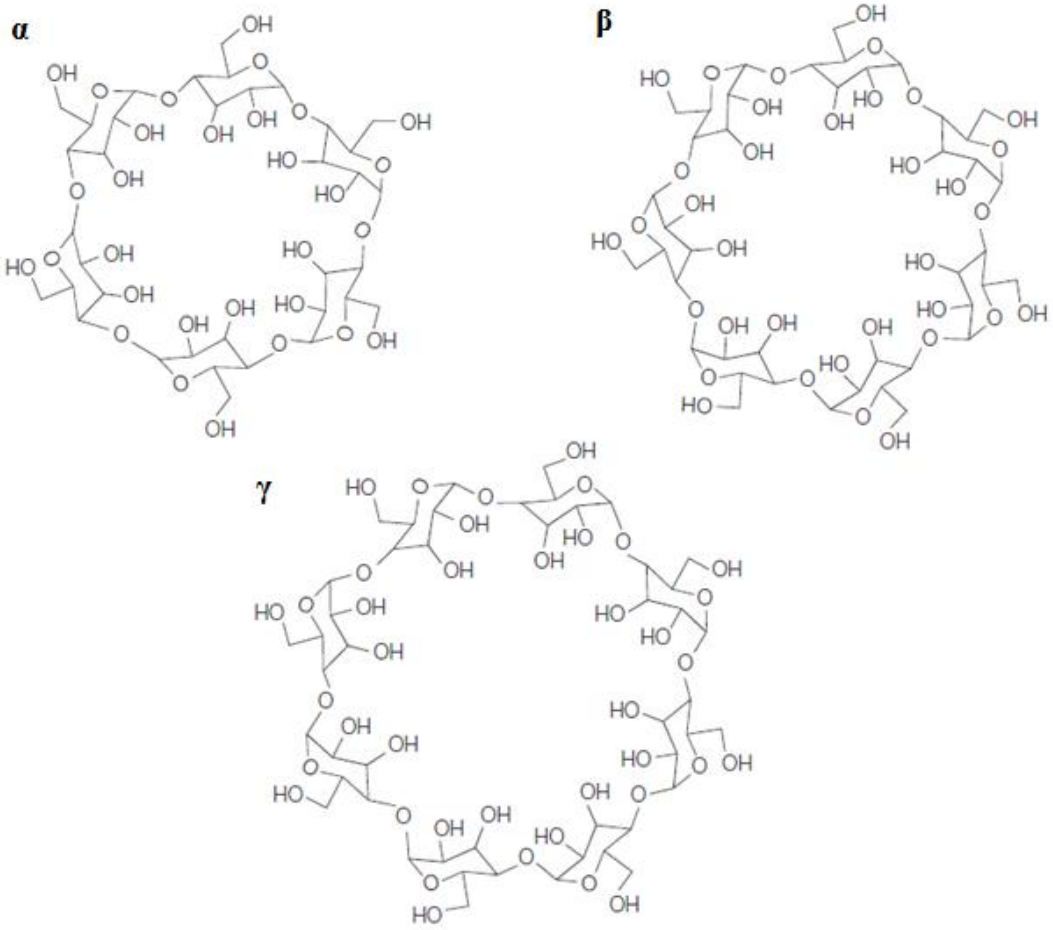

Figure 1: Chemical structure of α, β, and γ – Cyclodextrin. Modified from Davis and Brewster, 2004.

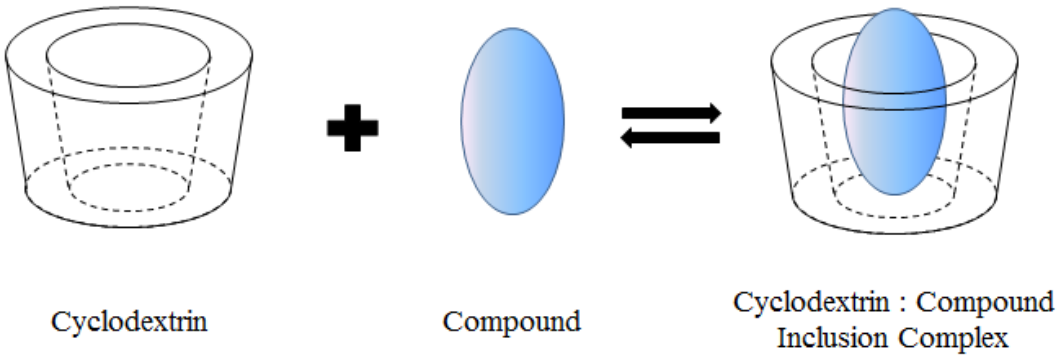

Figure 2: General illustration of free cyclodextrin and cyclodextrin:compound inclusion within the binding cavity. Modified from Davis and Brewster, 2004.

1.4.1 Cyclodextrin History

The history of cyclodextrin has been divided into three distinct time periods originally defined by József Szejtli in 1998. First was its discovery between 1891-1930, followed by systematic studies on cyclodextrins and their inclusions in 1930-1970, and finally the industrial production and utilization of cyclodextrins from 1970-present (Szejtli, 1998).

Cyclodextrin was first discovered by French microbiologist A. Villiers in 1891, when a he documented a crystalline material was formed from the digestions of starch by *Bacilus amylobacter* (Del Valle, 2004). Villiers isolated two distinct crystalline dextrins, named 'celluosine' due to the similarity in chemical properties to cellulose. In 1903, Schardinger incidentally discovered the same two crystalline dextrins, α and β – dextrin, while studying bacteria responsible for food poisoning. 'Schardinger's dextrins' were studied extensively for the next 30 years (Szejtli, 1998). The majority of research on these dextrins was completed by Pringsheim and colleagues, who discovered that these dextrin compounds can make inclusions with many other organic molecules (Szejtli, 1998). This discovery of the ability for dextrins to create inclusion complexes spurred the next era in cyclodextrin development.

In the 1930's, Freudenberg and colleagues discovered that 'Schardinger's dextrins' were comprised of cyclical α-1,4-glycosydic linkages. This discovery led to the renaming of 'Schardinger's dextrins' to the currently used name, 'cyclodextrins' (Szejtli, 1998). Freudenberg's group also discovered that cyclodextrins are created through the enzymatic mediated reactions involving starch subunits. Shortly after, γ-cyclodextrin was eluded by the same group of researchers (Szejtli, 1998). In 1953, Freudenberg obtained a

patent for cyclodextrins which outlined the many uses. Some of the early uses included the protection of easily oxidizable compounds, an increase in the solubility of poorly soluble compounds, and the reduction of loss of highly volatile compounds (Szejtli, 1998). In 1957 a study by A. French showed that cyclodextrin was highly toxic to rats. However, through inconsistencies and inability to replicate, it was determined that French's results were invalid (Szejtli, 1998). It is thought that the cyclodextrins used could have been contaminated (Szejtli, 1998). This however, was a major setback in the development and widespread use of cyclodextrins. It took 25 years for research to begin developing cyclodextrins for human consumption and industrial production, finally becoming a viable commodity (Szejtli, 1998).

From 1970 onwards, many conferences and symposiums were held describing the properties and usefulness of cyclodextrins. High awareness has led to industrial production and a subsequent reduction in cost from thousands of dollars to just a few dollars per kilogram (Szejtli, 1998). With reduced cost came widespread use and the cyclodextrin industry began booming in the mid-1990s. The highly desirable properties of cyclodexrins were being exploited very quickly. Soon after the start of industrial production, modifications were made to optimize their function. Of the more than 1500 cyclodextrin derivatives, hydroxypropyl-β-cyclodextrin (HPβCD) is one of the most widely used (Szente and Szejtli, 1999).

1.4.2 Cyclodextrin Properties

The cyclodextrin molecule has a cavity which allows it to form non-covalently bound inclusion complexes with 'guest' compounds (Figure 2). The cavity, being slightly apolar, is energetically unfavourable for water molecules to bind within due to polar-

apolar interactions (Szejtli, 1998). This slight hydrophobic trait allows apolar molecules to readily replace water molecules within the cavity while in an aqueous solution (Szejtli, 1998). Although most complexes are formed in a 1:1 cyclodextrin:guest ratio, 2:1 and 1:2 ratios are possible (Davis and Brewster, 2004). Inclusion often results in an increase in the aqueous solubility of the guest molecule. The increase in guest solubility is directly related to the solubility of the outer region of the cyclodextrin molecule and driven by thermodynamic favourability.

β-cyclodextrins are most commonly used due to their low cost of production and optimal cavity size for most applications, however they are not very water soluble (Table 1) (Szejtli, 1998). To increase the usefulness and application of β-cyclodextrin, hydroxylpropyl additions were made (Figure 3). These additions increased the polarity of the outer shell of the molecule resulting in an improvement of aqueous solubility. The result, HPβCD, has hydroxypropyl substituents on the hydroxyl sites on each of the seven glucopyranose units that make up β-cyclodextrin (Szente and Szejtli, 1999). The product is heterogeneous with one, two, or three hydroxypropyl substitutions (Szente and Szejtli, 1999). This addition dramatically enhances the water solubility and therefore the application of β-cyclodextrin. HPβCD can be described as being amphiphilic since it contains a hydrophobic inner cavity and a hydrophilic outer shell.

Table 1: Chemical properties and cavity sizes of α, β, and γ – Cyclodextrin. Modified from Szejtli, 1998.

	α	**β**	**γ**
Number of glucopyranose units	6	7	8
Molecular weight	972	1135	1297
Solubility in water, g 100 mL-1 at room temp	14.5	1.85	23.2
Cavity diameter Å	4.7-5.3	6.0-6.5	7.5-8.3
Height of torus Å	7.9 ± 0.1	7.9 ± 0.1	7.9 ± 0.1
Approximation of cavity volume $Å^3$	174	262	427

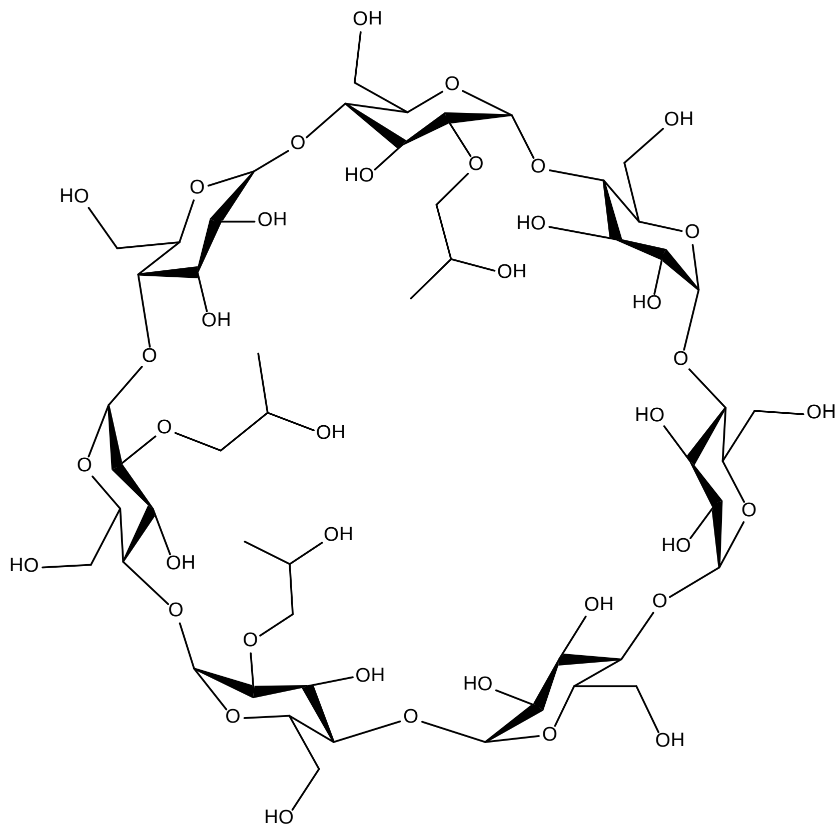

Figure 3: An example of the Chemical structure of hydroxypropyl-β-cyclodextrin. Modified from Szejtli, 1998.

1.4.3 Uses of HPβCD Globally and in Canada

Due to low specificity of guest inclusion, HPβCDs have wide uses both globally and in Canada. Currently, there are six major groups of large scale HPβCD use: personal care, food, chemical, environmental, agriculture, and pharmaceuticals (Del Valle, 2004).

One of the largest consumers of HPβCD is the personal care industry which often uses them as an odour suppressant. Through inclusion, HPβCD is able to trap and supress, or control, the release of volatile fragrances (Del Valle, 2004). Applications in personal care include toothpaste, creams, fabric softener, detergent, deodourant, and odour control sprays (Del Valle, 2004). Reducing foul odours and delaying the release of pleasant odours has allowed HPβCD to become a highly consumed product. Most uses of HPβCD in the personal care industry result in disposal into residential wastewater systems.

A highly emerging sector of HPβCD use is in the food industry as flavour protection and delivery. Many artificial flavours are highly volatile. Therefore, creating a complex with HPβCD allows for an effective method of flavour preservation (Del Valle, 2004). This allows many artificial and some natural flavours to be protected during freezing, cooking, and long term storage (Del Valle, 2004). Another highly sought after use of HPβCD is the removal of undesirable compounds in food, such as cholesterol. HPβCD is able to form a complex with cholesterol and improve the efficiency of its removal (Del Valle, 2004). For example, it has been shown that eggs and dairy products treated with HPβCD can experience up to 80% removal of cholesterol (Del Valle, 2004). Removal of cholesterol is seen as beneficial by most nutritionists and physicians as high levels of consumption have been linked to heart disease. Pressure from health experts has

driven the increased use of HPβCD in the food industry. Additionally, HPβCD has been shown to have the ability to remove free fatty acids from fats and oils, improving the physical cooking qualities (Hedges, 1998).

Cyclodextrins, including HPβCD, are extensively used in the chemical industry, functioning as catalysts, causing enantiomer separation, and for waste removal. Cyclodextrins are able to act as enzyme mimics, using the cavity to localize chemical reactions (Del Valle, 2004). HPβCD is also used in high performance liquid chromatography (HPLC) or gas chromatography (GC) in the stationary phase to help separate enantiomers (Del Valle, 2004).

HPβCDs ability to form complexes is also used in environmental sciences to remove organic compounds from soil, water and the atmosphere. When used in wastewater treatment, cyclodextrins have been shown to remove significant levels of harmful organic compounds (Del Valle, 2004). With the ability to create a complex with aromatic compounds such as benzene, phenols, as well as some insecticides. These inclusions may alter the environmental fate of the guest molecule. Additionally, HPβCD is proving to be an effective bioremediation tool (Hedges, 1998; Szetjili, 1998).

Due to its ability to create inclusions with pesticides, pheromones, and growth regulators, HPβCD is highly sought after in the agricultural industry (Del Valle, 2004). HPβCD acts to delay, control, and increase solubility of these compounds, effectively increasing their desired action (Del Valle, 2004). As a result of direct application to crops in a method similar to traditional pesticide use, there is a risk of environmental exposure.

Due to the amphiphilic properties of HPβCD, it is heavily used in the pharmaceutical industry (Loftsson and Brewster, 2011). For a drug to be successful, it most often has to be hydrophilic enough to reach the cellular membrane and hydrophobic enough to cross the cellular membrane. This balance in hydrophilic properties is difficult to obtain and is often the reason many drugs are not viable (Loftsson and Brewster, 2011). Since HPβCD has a hydrophilic outer region and hydrophobic cavity, it has been investigated and used as a drug carrier system. HPβCD acts to increase the bioavailability of a drug by improving the perceived solubility without physically altering the drug (Hedges, 1998; Del Valle, 2004). Due to the fact that HPβCD is a large, water soluble molecule, it is able to reach, but not penetrate the cellular membrane. However, a non-covalently included drug molecule is able to readily disassociate from HPβCD at the cellular membrane, increasing the amount of drug available to enter the cell (Rasheed *et al.*, 2008).

The use of HPβCD has resulted in the use of drugs that would otherwise not be able to reach cellular membranes. Additionally, by including drugs within HPβCD, a lower dose is often required to achieve similar effect level (Szejtli, 1997). Another advantage of the use of HPβCD in pharmaceuticals is that they act to reduce the volatility of a drug through inclusion (Szejtli, 1997). This may allow for some drugs to be administered as a non-invasive pill rather than an injection (Szejtli, 1997).

1.4.4 Environmental Fate of HPβCD

It is very important to understand the environmental fate of a pollutant when determining its potential environmental impact. Understanding where a compound is most likely to partition, degrade, or persist is vital to determining where it may impart its

impacts. There are four major locations for a compound to become localized within an ecosystem: the aquatic environment, soil, atmosphere, and within biota.

With most HPβCD research involving applications and human safety, very little work has been completed regarding potential environmental impacts. Due to this lack of research, the full environmental fate of HPβCD is presently unknown. However, based on HPβCD's chemical properties, a generalized environmental fate can be predicted.

One of the most important factors in determining the fate and localization of a chemical is how hydrophilic the compound is. If a compound is hydrophilic, there is an increased likelihood that it will end up in the aqueous phase of the environment. Given that HPβCD is very hydrophilic, the likelihood of it partitioning into the aquatic environment is high. Additionally, low volatility of HPβCD means that it is not likely to sequester within the atmosphere. Finally, being highly water soluble, HPβCD is not likely to partition into lipids, but rather water, thereby reducing the possibility of bioaccumulation within biota (Simonich and Hites, 1995).

Given that HPβCD is most likely to partition within the aquatic phase of the environment, it is important to determine its biodegradability. Cyclodextrin biodegradability is inhibited with increasing number of substitutions (Yuan and Jin, 2007). This effect is most likely attributed to the steric hindrances of both the cyclic structure and the substituted side chains on the enzymatic degradation of HPβCD (Szente and Szejtli, 1999; Yuan and Jin, 2007).

1.4.5 Toxicity of HPβCD

The toxicity of HPβCD has been well documented in many mammalian models due to the large number of human applications. In the model species tested, HPβCD is for the most part, toxicologically benign (Gould and Scott, 2005). This apparent lack of significant toxicity, both acute and chronic, in mammalian models has permitted HPβCD to be used in the food and pharmaceutical industries (Gould and Scott, 2005). However, there is a paucity of toxicological information regarding HPβCDs impact within the environment, something that is of growing concern.

In acute mammalian studies on rats, HPβCD did not cause any significant toxicity at intra-peritoneal doses as high as 10 000 mg/kg of body weight (Gould and Scott, 2005). Chronically, HPβCD has shown only minor toxicological responses. Chronic intravenous exposure in mice showed minor histopathological changes in the lungs, liver and kidneys, all of which were reversible (Gould and Scott, 2005).

When HPβCD was orally dosed in rats, minimal toxicity was observed. This low toxicity has been attributed to a few key points; HPβCD experiences little absorption across the intestinal tract, has low membrane permeability, and only a small fraction of HPβCD is metabolized in the colon in a similar fashion to starch (Gould and Scott, 2005). However, the hydroxypropyl side chains combined with the cyclic nature of HPβCD makes enzymatic degradation more difficult, resulting in slower and less efficient metabolism than starch (Gould and Scott, 2005).

Most mammalian studies have looked at a large scale picture of the toxicological impacts, with little effects being seen. However, one major toxicological impact of

HPβCD is depletion of cholesterol in both blood and cellular membranes (Hui *et al.*, 2011). A dose-dependent response in cell viability, as well as an increase in low-density lipoprotein in human embryonic kidney cell lines has also been reported (Hui *et al.*, 2011). Cholesterol depletion has been investigated as a marker for nephrotoxicity for pharmaceutical application (Hui *et al.,* 2011). A small change in membrane cholesterol levels may alter the structural integrity as well as the membrane fluidity. This can influence cell signalling and cell function, thereby potentially limiting the effectiveness of cells exposed to HPβCD (Gould and Scott, 2011).

Although mammalian research has been undertaken, both the environmental impact and the reproductive toxicity of HPβCD have not been fully investigated in aquatic ecosystems. Importantly, HPβCD has been shown to create complexes with many important reproductive hormones including testosterone, progesterone, and estradiol (Taylor *et al.,* 1989; Hoon *et al.,* 1993; Zoppetti *et al.,* 2007). The result may be an increase in the bioavailability of these compounds through inclusion. Given that HPβCD does not readily cross the cellular membrane, alteration of hormone availability, positively or negatively, may act as a significant route of toxicity, possibly through endocrine disruption. This route of toxicological impact has not yet been investigated on aquatic organisms; as chronic exposure has the potential to alter the availability of reproductive hormones, such investigations should be undertaken.

1.5 Aquatic Toxicology and Testing

1.5.1 Acute Toxicity

Acute toxicity is defined as a stimulus that is severe enough to cause a response within a short period of time, often 96 h for fish (Sprague, 1969). The most unambiguous endpoint of acute toxicity is lethality, often denoted as an LC_{50}, which is the concentration that causes 50% mortality over a specified time period, generally 96 h. Alternately to using LC_{50}, the effective concentration, EC_{50}, can be measured by determining the concentration at which a toxicant is able to produce a biological response in 50% of the test organisms. Although chosen arbitrarily, LC_{50}/EC_{50} values are very important in that they provide a reference point for the acute toxicity of the compound of interest.

1.5.2 Chronic Toxicity

Chronic toxicity measures the effects of prolonged exposure to a compound, usually defined as greater than one tenth of an organism's life cycle (Sprague, 1973). Chronic toxicity can also refer to the long lasting effects of a short exposure, such as a pulse dose. The endpoints of chronic exposure studies are often more numerous than acute studies. For example, if one is interested in reproduction, appropriate endpoints would include, egg output, egg viability and time to spawn (Sprague, 1973). On the other hand, when general health condition is of interest, length, weight, condition factor, hepatosomatic index (HSI), and gonadosomatic index (GSI) of the organism are determined.

1.6 Fish Physiology

1.6.1 General Fish Absorption and Elimination Physiology of Toxicants

The physiological processes which occur when a toxicant enters the body can be described in four major steps: absorption, distribution, metabolism, and elimination, abbreviated to ADME (Leeson and Springthorpe, 2007). Absorption of toxicants in fish can occur through sediment-borne, food-borne, or water-borne exposure (Nichols *et al.*, 2007). The most common routes of absorption into the body include oral, consumption of contaminated sediment or food, or dermal, via direct absorption through the gills or skin (van der Oost *et al*., 2003). The rate and efficiency of absorption is often toxicant dependant. Absorption rates can also be impacted by modifying factors such as temperature, pH, water hardness, species, age, and gender (Lewis, 1992).

Once within the body, internal distribution of the contaminant occurs. After oral ingestion the compound can be either absorbed by the gastrointestinal epithelium and enter the blood stream, or adsorb to the contents of the gut (Nichols *et al.*, 2007). If the latter occurs, direct elimination is possible. If the former occurs, distribution to the site of action can occur. If a compound is absorbed dermally, the toxicant can often readily enter the bloodstream and be delivered to the site of action (Nichols *et al.*, 2007). It is the site of action where the toxicant can elicit an effect.

Metabolism of the toxicant often occurs after delivery to the site of action. The goal of metabolism is to allow for more efficient elimination. As a general rule, the more hydrophilic a compound is, the easier for elimination to occur. Metabolism and biotransformation most often occurs within the liver. The addition of bulky substituents

such as glutathione increases solubility, promoting elimination. While metabolism often promotes elimination, it has the potential to increase the toxicity of a given compound relative to the parent (van der Oost *et al*., 2003).

Elimination occurs through two main routes, urine and feces (Nichols *et al.*, 2007). Elimination is greatly impacted by the hydrophobicity of the toxicant and often occurs readily if the compound of interest is hydrophilic. However, if a compound is hydrophobic, there is a greater tendency for the compound to become sequestered within the lipid tissue of the fish (van der Oost *et al*., 2003). If sequestration occurs, the toxicant may remain trapped until the lipid tissue is consumed. Consumption of this contaminated tissue can cause the organism to be re-exposed to the initial toxicant.

1.6.2 General Fish Reproductive Physiology

Reproduction in fish, as in many organisms, is complicated, relying on both external and internal stimuli to elicit a response (Kime, 1995; Kime, 1999). External stimuli are often the initial trigger in the reproductive process. Seasonal variation including temperature, photoperiod, and precipitation change are the most common triggers (Kime, 1995; Kime, 1999). Once an external stimulus is experienced by fish, an internal signal cascade may follow. Firstly, gonadotrophin releasing hormone, emanating from the hypothalamus, causes the pituitary gland to release gonadotrophin (Kime, 1995). Gonadotrophin results in the initial development of the gonads and the production of steroid hormones in both males and females (Kime, 1995). In males, the key androgenic hormone is 11-ketotestosterone, mostly produced in the testes; in females the key estrogenic hormone is known as estradiol, the majority originating in the ovaries (Kime, 1995). These reproductive steroids maintain gonadal development. The secretion of 11-

ketotestosterone and estradiol eventually subsides and progesterone is then produced (Kime, 1995). Progesterone initiates the maturation of the sperm and oocyte in the males and females respectively (Kime, 1995). Maturation in females involves the expression of vitellogenin, a yolk precursor protein, vital for the production of yolk with in the egg (Kime, 1995).

1.7 American Flagfish (*Jordanella floridae*)

1.7.1 Characteristics and Habitat

Jordanella floridae, commonly known as the American or Florida flagfish, is a member of the *Cyprinodontid* family (Foster *et al.*, 1969). They are native to shallow weedy freshwater regions surrounding the Gulf of Mexico (Foster *et al.,* 1969). Flagfish have a short lifecycle, reaching maturity between 90 and 120 days after hatch depending on water temperature and feeding regime (Holdway and Dixon, 1986). Flagfish can reach a maximum length of 50 mm and 45 mm for males and females respectively (Foster *et al.,* 1969).

1.7.2 Behaviour and Breeding Habits

Flagfish are able to reproduce with consistently high output at any time of the year, provided they have the correct photoperiod, temperature and feeding regime. In ideal situations, flagfish prefer to breed in water temperatures between 25 - 26º C, with a 16 h light and 8 h dark photoperiod (Foster *et al.*, 1969). When breeding, the male flagfish tends to the eggs and guards the nest which often consists of an algae covered surface such as a rock. While guarding the nest from other males, female flagfish will approach the guarding male and commence a breeding “t-dance”. The female positions

her body perpendicularly, tail first, to the side of the male. This occurs for a short period of time before they align themselves head to head and tail to tail allowing the female to expel eggs while the male fertilizes them. The male continues to guard the nest and tends to the eggs by fanning, cleaning and protecting the eggs until they hatch, usually between five and seven days after fertilization, dependant on temperature (Klug *et al.*, 2003).

In a laboratory setting, a breeding harem is often set up involving two males and four or five females. The males will compete for dominance, and only one will become the dominant breeding male. Females approach the dominant male, breed, laying their eggs on the nest. A nest in a laboratory setting is often a glass plate wrapped in green Orlon® wool, mimicking a moss covered surface. The nest is removable for ease of egg collection. In laboratory breeding harems, the American flagfish can produce between 100-200 eggs per day, sometime more (Beyger *et al.*, 2012).

1.7.3 American Flagfish as a Test Species

Fish are ubiquitous in nearly all aquatic ecosystems and thus utilizing fish as test species for assessing chemical toxicity is very important. Fish provide humans with a valuable food source, and are an integral part of many ecosystems, including terrestrial. Understanding the toxicity of a compound to fish will ultimately help protect ecosystem function and, potentially, human health. However, not all fish are good test species and a few characteristics can determine their usefulness. Test organisms should ideally be representative of the native fish species which are found in the contaminated waters (Fogels and Sprague, 1977). Secondly, test species should have a relatively quick lifecycle as a rapid lifecycle permits the examination of the effects of the compound over

an entire lifecycle. Finally, they should be relatively easy to maintain and quick to breed in the laboratory (Fogels and Sprague, 1977).

The American flagfish fits the description of a good model test organism very well. As stated above, they are found in shallow freshwater areas in North America and have shown similar responses to toxicant stressors commonly found in many fish in North America (Foster *et al.*, 1969; Fogels and Sprague, 1977). Also, with their ability to reproduce in 3-4 months, a full lifecycle study is possible. Finally, their small size and their high daily egg output mean they do not require a large amount of laboratory space to observe reproductive effects.

1.7.4 Use and Sensitivity in Literature

The American flagfish has been used in many previous aquatic toxicological studies. Studies have been published using flagfish for both acute and chronic studies which include pulse and continuous exposure (Holdway *et al*., 1982; Holdway and Dixon, 1986; Beyger *et al.*, 2012). Furthermore, when compared to other model fish species, flagfish display similar responses to many toxicants (Fogels and Sprague, 1977; Holdway and Dixon, 1986).

1.8 Knowledge Gaps

Although HPβCD is generally safe for human consumption, the impact on aquatic environments has not yet been fully investigated. Of particular interest is the impact HPβCD has on the survival and reproduction of biota within aquatic ecosystems.

2.0 Rationale and Research Objectives

2.1 Rationale

This research is required in order to characterize the potential effects that HPβCD may be having on non-target organisms, particularly fish. Very little research has examined the non-target effects of HPβCD in aquatic environments. HPβCD has the ability to include important signalling molecules, such as hormones within its binding cavity (Taylor *et al.*, 1989; Hoon *et al.*, 1993; Zoppetti *et al.*, 2007). This non-specific inclusion affinity for hormones as well as high usage makes HPβCD a compound of interest in the field of aquatic toxicology. To understand the influences of HPβCD on biological systems at environmentally relevant conditions both an acute and chronic exposure should take place. Importantly, a full life-cycle study should be undertaken, allowing for exposure at all life-stages. Given that HPβCD is used in many different processes with the potential to enter aquatic ecosystems, the toxicological effects on a representative fish species should be determined.

2.2 Assess the Acute Toxicity of HPβCD

The first objective of this study was to assess the acute toxicity of HPβCD to the American flagfish. This was done by undertaking a standard 96-h LC_{50} bioassay on larval flagfish. This approach was used to provide a standard measure of the relative toxicity of HPβCD to the flagfish.

2.3 Assess the Chronic Toxicity of HPβCD

The second objective was to determine the impacts of chronic HPβCD exposure on flagfish over one full life-cycle. This information would provide important data regarding the growth, reproductive, and survival abilities of flagfish exposed to HPβCD.

2.4 Assess Offspring Sensitivity to a Reference Toxicant after Parental HPβCD Exposure

The third and final objective of this project was to determine if there were any differences in sensitivity to copper sulphate ($CuSO_4$), a known reference toxicant, on larval flagfish whose parents were chronically exposed to HPβCD.

2.5 Hypothesis

The null hypothesis for this project states: there are no effects of HPβCD exposure over one life-cycle on the reproduction, growth, survival, or sensitivity to a reference toxicant on the American flagfish.

3.0 Materials and Methods

3.1 Laboratory Organism Maintenance

American flagfish (*Jordanella floridae)* where reared and maintained with their offspring being used in all subsequent studies. The fish were housed in multiple 70 L flow-through glass aquaria containing an air stone for circulation and aeration and plastic greenery as environmental enrichment. The water was maintained at 25.0 ± 1.0 ºC with a photoperiod of 16 hours light and 8 hours dark, including a half hour of dawn and dusk. All aquaria were covered with a transparent plastic lid.

3.1.1 Feed

Feeding consisted of three types of food throughout the experiment: flake food, frozen brine shrimp and freshly hatched brine shrimp. Flake food, specifically, Tetramin™ *Pro Flake* (Tetra United Pet Group), contained a minimum crude protein of 46.0 %, 12.0 % crude fat, 3.0 % crude fibre, 1.1 % phosphorus, 200 mg/kg ascorbic acid, and maximum moisture of 8.0 %. *Bio-Pure* frozen brine shrimp was purchased from *Hikari Sales* (*Hayward, California*) and consisted of 8.0 % minimum crude protein, 5.0 % minimum crude fat, 2.0 % maximum crude fiber, and 86.0 % maximum moisture. Premium brine shrimp eggs were purchased from Brine Shrimp Direct (*Ogden, Utah*) and were harvested daily as first instar nauplii.

3.1.2 Breeding, Egg Collection, and Larval Rearing

Breeding harems were selected from the flagfish stocks maintained in section 3.1 to include two male and four female flagfish. Fish selection was based on size; two

males, one larger than the other, and four females that were smaller than the males. A differential in male size allows for easy establishment of dominance. All fish selected were in good health based on fin condition and overall colouration. The breeding harems were housed in separate 70 L glass flow-through aquaria that contained an air stone and a breeding substrate. The breeding substrate was placed in a central location in the tank and consisted of a 15.0 cm x 10.0 cm x 0.7 cm glass plate, wrapped in green Orlon® wool, mimicking an algae covered surface. Feeding consisted of freshly hatched first instar brine shrimp nauplii and frozen adult brine shrimp three times daily, and crushed flake food once daily.

Breeding substrates were removed daily from each aquarium using a set of modified ice tongs. All eggs were brushed off into separate 1 L polypropylene container containing 25º C laboratory water. The eggs were then placed in a temperature control room (27.0º C). To prevent contamination between aquaria tongs were dipped into a 1.0 % w/v Virkon solution, rinsed with 25º C lab water and 70 % ethanol after each aquarium. Also, breeding substrates were immediately replaced into the same aquarium after eggs were collected. Eggs were then counted and transferred to 25 mL petri dishes containing rearing solution. Rearing solution was made in house and comprised of 10 % NaCl, 0.30 % KCl, 0.40 % $CaCl_2 \cdot 2H_2O$, 1.63 % $MgSO_4 \cdot 7H_2O$, 0.01 % methylene blue, and distilled water. All eggs remained in rearing solution until hatch, lessening the presence of fungus on the eggs.

After hatch, rearing solution was replaced with 25º C laboratory water. Once the yolk sacs were fully consumed, the larval flagfish were transferred into polypropylene containers that contained 1 L of 25º C laboratory water. After being transferred, the larval

flagfish were fed freshly hatched brine shrimp nauplii twice daily. Each container underwent daily siphoning of uneaten food and a 95 % solution change. Once they were large enough, approximately 1 cm in length, the flagfish were transferred to 70 L aquaria.

3.2 Chemicals and Chemical Preparation

Hydroxyproply-β-cyclodextrin (HPβCD), 97.0 %, was purchased from Acros Oganics (*Thermo-Fisher Scientific*). HPβCD solutions were prepared by weighing the required mass and dissolving into 1 L of distilled water to be used as a stock solution. The stock solution for the flow-through portion of this experiment consisted of 12.3 g/L HPβCD in distilled water. Serial dilutions were then made in order to obtain desired working HPβCD stocks of 0, 3.93, 1.23, 0.39, 0.12, 0.04, and 0.01 g/L. A peristaltic pump delivered working stocks of HPβCD to aquaria at a rate of 80 µL/min. Each tank contained 56.6 L of water, which underwent five turnovers per day. The resulting concentrations within the aquaria were, 0, 5, 16, 50, 160, 500, and 1600 µg/L.

Copper sulphate (*Thermo-Fisher Scientific*) was weighed and dissolved into 25º C laboratory water to be used as a stock, and subsequent solutions were made through serial dilutions. Based on molecular weight, calculations were made such that the desired concentration was a representation of copper content rather than copper sulphate content.

Tricaine methanesulfonate (MS-222) (*Western Chemical*) and sodium bicarbonate (*Sigma-Aldrich*) were used as an anaesthetic and a buffering agent in a 1:2 ratio respectfully. A concentration of 350 µg/L of MS-222 and 700 µg/L sodium bicarbonate were used. Both chemicals were weighed and dissolved in into 750 ml of 25º C laboratory water to euthanize the flagfish.

3.3 Water Parameters

3.3.1 Temperature and pH

The pH of the 25º C general laboratory water was monitored and recorded daily using a SevenEasy pH meter (*Mettler-Toledo*). Temperature readings for each aquarium were recorded daily using a Traceable® infrared thermometer.

3.3.2 Water Hardness, Alkalinity, Nitrite, and Nitrate

All Purpose 5-way Test Strips from Lifeguard Aquatics (*Cerritos, California*) were used to determine water hardness, alkalinity, nitrite, and nitrate in each aquarium. As per product instructions, the strip was quickly dipped into the aquarium swirled two times. Without shaking off excess water, water hardness, alkalinity, and pH were immediately read, nitrite and nitrate were read 30 seconds after dipping the test strip. For each parameter, the strip was compared to a colour chart with corresponding reference values. Water hardness, alkalinity, nitrite, and nitrite levels were measured once at 110 DPH.

3.3.3 HPβCD Concentration

A collection of 1 L samples from each aquarium were analyzed to determine actual HPβCD concentration within each 70 L flow-through aquaria (Water Quality Center, *Trent University, Peterborough, Ontario*). Results of actual HPβCD concentrations in the flow through treatment aquaria are pending. As such, all HPβCD values stated are nominal concentrations.

3.4 Cleaning and Sterilization of Equipment

Prior to use, all glassware and 1 L polypropylene containers underwent a cleaning cycle in a dishwasher using Neodisher® detergent (*Dr. Weigert*). Additionally, all equipment was sterilized using a 70 % ethanol solution before and after use with the exception of nets and breeding substrate extraction tongs. These items were also soaked in a 1.0 % w/v Virkon® solution (Vétoquinol N.-A. Inc, *Lavaltrie, Quebec*) and thoroughly rinsed with 25º C laboratory water to ensure sterility.

3.5 Acute Larval 96-h Continuous Exposure Study

American flagfish eggs were collected and reared as described in section 3.1.2 from five separate breeding aquaria. All eggs were pooled and healthy 2-day post hatch (DPH) larval flagfish were randomly placed into petri dish containing 20 mL of control water or HPβCD treatment. Treatments concentrations consisted of: 0 (control), 1, 10, 100, 1000, and 10 000 µg/L HPβCD. There were 10 fish per petri dish and each treatment had two replicates for a total of 120 larval flagfish. Each petri dish underwent a 95 % static renewal once every 24 hours for the duration of the 96 hour exposure. All solution changes were completed from low to high HPβCD concentration, minimizing risk of unintended exposure. The fish were starved for the entirety of the experiment. Temperature was monitored as per section 3.3.1 for each petri dish. Mortality was monitored at 0.5, 1, 2, 4, 8, 16, 24, 48, 72, and 96 hours after initial exposure. A photoperiod of 16 hours light and 8 hours dark with half hour of dawn and dusk included in the light phase was used. Holding all other parameters the same, this experiment was repeated at higher HPβCD concentrations. Treatments consisted of 0 (control), 1, 10, 100, 1000 mg/L.

3.6 Chronic Life-Cycle Continuous Exposure Study

3.6.1 Egg Collection and Initial Exposure

American flagfish eggs were collected as described in section 3.1.2 from five separate breeding aquaria. These eggs were pooled and randomly placed into a petri dish containing a HPβCD treatment and rearing solution. The treatments consisted of: 0 (control), 5, 16, 50, 160, 500, and 1600 µg/L HPβCD. There were 30 flagfish per replicate with two replicates for each HPβCD concentration and control, totalling 60 fish per treatment. The rearing solution concentration was consistent among the control and each of the HPβCD treatments. The control water consisted of 25º C laboratory water and each treatment underwent a daily 95 % solution change. All solution changes were completed from low to high HPβCD concentration, minimizing risk of unintended HPβCD exposure. All treatments were maintained within a 27.0º C temperature control room and were randomly placed on the bench top. A photoperiod of 16 hours light and 8 hours dark with half hour of dawn and dusk included in the light phase was maintained for the duration of the life-cycle study.

3.6.2 Transfer to 1 L Polypropylene Containers

After hatch, the use of rearing solution ceased and the fish were transferred to polypropylene containers containing 1 L of their respective HPβCD or control solutions. HPβCD concentrations were maintained with 95 % daily renewal of solution from low to high HPβCD concentration. A transparent acrylic lid was placed on top of the containers to minimize evaporation and to aid in temperature regulation. All treatments were maintained within a 27.0º C temperature control room and were randomly placed on the

bench top. Twice daily feeding of freshly hatched brine shrimp nauplii also began at this time.

3.6.3 Length Measurements, 21 and 28 DPH

At 21 and 28 days post hatch (DPH), non-lethal growth measurements were collected. Photographs were taken of each of the 1 L polypropylene containers containing the juvenile fish using a Canon® 40D DSLR. A bar of known measurement was placed within the container to calibrate an image analysis program, ImageJ (National Institutes of Health). After calibration, fish lengths were measured using the measurement function in ImageJ. Only fish that had focused heads and tails, and were near the bottom of the container were measured.

3.6.4 Transfer to 70 L Flow-Through Aquaria

At 30 DPH, fish were transferred out of the temperature control room and into 70 L glass aquaria containing 65 L of water. Each flow-through aquaria experienced a 5 x solution turnover daily, resulting in a 99 % molecular turnover in a 24 hour period (Sprague, 1969). HPβCD concentrations were maintained using a *Watson-Marlow 200 Series* 16 channel peristaltic pump (Watson-Marlow). The pump delivered the appropriate amount of HPβCD from individual 1 L working stock bottles to each aquarium through a Y-connecter attached to the inflowing water tube. The concentration of each working HPβCD stock bottle was determined based on the peristaltic pump flow rate, the water flow entering the aquarium, and the desired HPβCD concentration in the aquarium. Each working HPβCD stock was created from a central stock. Working stocks were remade and bottles were refilled once every 9 days. Temperature recordings, as well as siphoning of uneaten food and feces from each aquarium occurred daily. pH was also

measured daily as stated in section 3.3.1. Sterilization occurred as per section 3.4 to minimize cross contamination and accidental exposure to HPβCD. Feeding of freshly hatched brine shrimp nauplii three times daily, and crushed flake food once daily occurred during this stage.

3.6.5 Initial Thinning

From 75 to 80 DPH each aquarium was thinned down to 15 fish in order to lower tank density and to account for any mortality. The removed fish were euthanized using 350 mg/L of MS-222. Total length and wet weight were determined using an electronic caliper with digital display and an AB204-S analytical balance (Mettler-Toledo). Hepatosomatic index (HSI) and condition factor (K) were calculated using the following formulas:

$$HSI = (W_l \div W_f) \times 100$$

Where W_l is the wet weight of the liver in grams and W_f is the wet weight of the fish in grams (Sadekarpawar and Parikh, 2013).

$$Condition\ Factor\ (K) = (W \div L^3) \times 100$$

Where W is the wet weight in milligrams of the fish and L is the total length in millimeters of the fish. (Smolders *et al*., 2002)

3.6.6 Second Thinning

From 101 to 103 DPH each aquarium was thinned down to breeding harems including 2 males and 4 females. Removed fish were euthanized using 350 mg/L of MS-222. Total length and wet weight were measured. Hepatosomatic index (HSI) and condition factor (K) were calculated using the formulas found in section 3.6.5, while gonadosomatic index was calculated using the following formula:

$$GSI = (W_g \div W_f) \times 100$$

Where W_g is the wet weight of the gonads in grams and the W_f is the wet weight of the fish in grams (Sadekarpawar and Parikh, 2013).

3.6.7 Breeding, Egg Collection, and Second Generation Rearing

After thinning down to the breeding harem, a breeding substrate was added to each 70 L aquarium. Breeding aquaria were setup and fed as stated in section 3.1.2. General maintenance remained unchanged from section 3.6.4. Egg collection occurred for 30 days after the setup of the breeding harem.

All eggs were collected daily, counted, and raised in 25 mL petri dishes containing only rearing solution. Egg production and egg quality was monitored for each treatment for the first 28 days of the breeding period. Production parameters included: time to first egg laying, defined as how many DPH the parents required to produce an egg, time to steady state spawning, defined as how many DPH the parents required to produce greater than 30 eggs per day for four consecutive days, the total number eggs, and the total number of days eggs were produced during the 28 day breeding period. The

mean daily egg production was also determined, defined as the total number of eggs produced divided by the number of days eggs were produced.

The quality of eggs was also monitored from each treatment. Quality was determined by monitoring time-to-hatch, the number of days required to hatch, percent hatch, percent fertilized, and percent malformed. All larval fish were euthanized and discarded after hatch except for the eggs collected on days 29 and 30 of the breeding period.

The eggs collected on days 29 and 30 of the breeding period were pooled and treated with HPβCD in the same fashion as their parents, described in sections 3.6.1 and 3.6.2. Length measurements were made at 21 and 28 DPH as described in section 3.6.3. Mortality was also monitored. All second generation fish were euthanized at 30 DPH.

3.6.8 Larval Copper Challenge

Larval flagfish from each treatment aquaria were obtained on day 28 of the breeding period, and replicate treatments were pooled after hatch. Copper 96-h LC_{50} values were determined using the offspring of the parents exposed to various concentrations of HPβCD or 25º C control lab water. The LC_{50} experiment was run in triplicate with six concentrations of copper, 0 (control), 2.5, 5, 10, 20, and 40 µg/L, with 15 larval flagfish per treatment. Solutions were prepared as described in section 3.2. Each treatment underwent a 95 % daily static solution change. All fish were unfed during this study. Lethality of the offspring from each HPβCD were compared to the control to determine any change in sensitivity to a reference toxicant. Any surviving flagfish were euthanized and discarded.

3.6.9 Final Dissection

On days 144 and 145 DPH, all breeding flagfish were euthanized and dissected. Total length, wet weight, wet liver weight and wet gonad weight were determined. Also, condition factor, HSI, and GSI were all calculated using the formulas found in sections 3.6.5 and 3.6.6.

3.7 Statistics

Statistical analysis was completed using the *Sigmaplot 12.0* software package (Statsoft, Inc. *San Jose, USA*). Data from the full life-cycle study, section 3.6, was tested for normality using the Shapiro-Wilk's W test, and homogeneity of variance was tested using Levene's test ($P \leq 0.05$) prior to running parametric statistics. Additionally, to test if replicates could be pooled, a one way ANOVA (analysis of variance) was completed. If replicates were not significantly different ($P \leq 0.05$), they were pooled. Next, the treatments underwent a one way ANOVA ($P \leq 0.05$) and Tukey post-hoc analysis to determine effects.

4.0 Results

4.1 Acute Larval 96-h Continuous Exposure Results

4.1.1 Abiotic Factors

There were no significant differences in temperatures between HPβCD treatments during acute larval toxicity testing ($P \leq 0.05$). An overall average temperature of 26.4 ± 0.3º C was experienced.

4.1.2 Acute Toxicity of HPβCD

Very little mortality was observed during the 96-h acute exposure studies. There was one death in both the control and 1 µg/L treatments over the first acute exposure experiment including concentrations up to 10 mg/L HPβCD. No mortality was observed in any other treatment. Similarly, no mortality occurred in the 96-h exposure up to and including 1 g/L HPβCD.

4.2 Chronic Life-Cycle Continuous Exposure Results

4.2.1 Abiotic Factors

There were no significant differences in temperatures between HPβCD treatments during the chronic life-cycle experiment ($P \leq 0.05$), with an overall average temperature of 26.2 ± 0.5º C. The pH of the lab water was monitored daily and averaged 7.71 ± 0.03 (Table 2).

There were no significant differences between water hardness, alkalinity, nitrate, and nitrite levels between tanks when sampled at 110 DPH (Table 2).

Table 2: Abiotic factors measured during the chronic life-cycle study exposing American Flagfish to HPβCD. Values are pooled from each tank and reported as means ± SE.

Abiotic Factor	**Mean ± SE**
Temperature (ºC)	26.2 ± 0.5
pH	7.71 ± 0.03
Water Hardness (ppm)	45
Alkalinity (ppm)	35
Nitrate (ppm)	6
Nitrite (ppm)	0 – 0.5

4.2.2 Mortality

Limited mortality was observed over the course of the life-cycle study. Percent mortality was recorded and none of the HPβCD treatments were significantly different from the control ($P \leq 0.05$) (Table 3).

Table 3: The impact of chronic exposure to varying concentrations of HPβCD over a complete life-cycle on the survival of the American flagfish. Both replicates were pooled for each treatment (n=60) and values are given as means ± SE. There was no statistically significant difference between treatments ($P \leq 0.05$).

HPβCD Treatment (µg/L)	**n**	**% Mortality**
0 – Control	60	8.3 ± 5.0
5	60	3.3 ± 3.3
16	60	8.3 ± 5.0
50	60	8.3 ± 1.7
160	60	5.0 ± 1.7
500	60	3.3 ± 0.0
1600	60	6.7 ± 0.0

4.2.3 Growth

There was no significant difference in length between replicates at 21 DPH thus replicates were pooled and reported as means ± SE ($P \leq 0.05$). Fish exposed to 50 µg/L of HPβCD were significantly larger than the control, 16, 160, and 500 µg/L HPβCD treatments ($P \leq 0.05$) (Figure 3).

Length at 28 DPH was not different between replicates and thus replicates were pooled and reported as means ± SE ($P \leq 0.05$). There were no significant differences in length between HPβCD treatments and controls ($P \leq 0.05$) (Figure 4).

Third, fourth, and fifth growth measurements were taken during sampling at 74-77, 102-103, and 144-145 DPH respectively, separated by gender, and analyzed based on both total length and wet weight. There were no significant differences ($P \leq 0.05$) between replicates at each time point for either total length or wet weight, and therefore, replicates were pooled for further analysis.

Total length and wet weight of both males and females at 74-77 DPH were not significantly different ($P \leq 0.05$) between HPβCD treatments and controls. Female flagfish from the 500 µg/L HPβCD treatments weighed significantly less than the 50, 160, and 1600 µg/L HPβCD treatments ($P \leq 0.05$) (Figures 5 and 6).

There were no significant differences in total length and wet weight at 102-103 DPH between HPβCD treatments and the controls ($P \leq 0.05$) (Figures 7 and 8).

Similarly, no significant differences in total length and wet weight between the HPβCD treatments and controls were observed after thirty consecutive days of breeding at 144-145 DPH ($P \leq 0.05$) (Figures 9 and10).

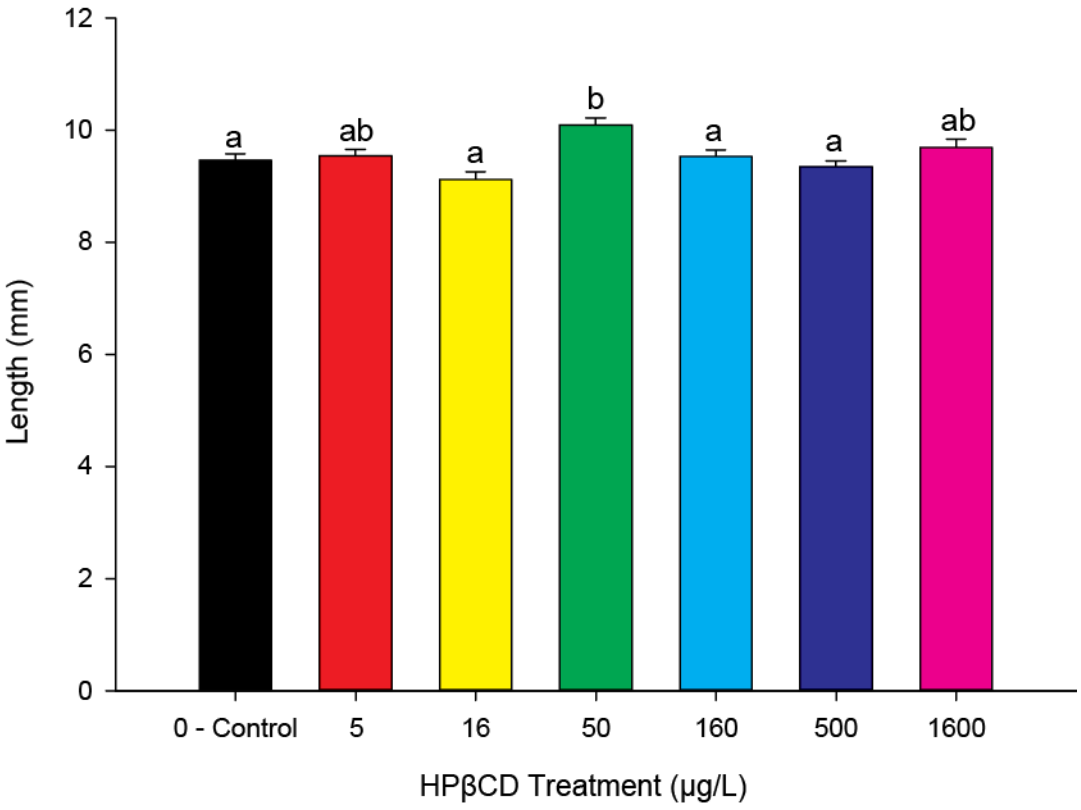

Figure 4: Total Length (mm) of 21 day old flagfish exposed to varying concentrations of HPβCD. Replicate data were pooled and reported as means ± SE. Bars without a letter in common are significantly different ($P \leq 0.05$). n=42-58.

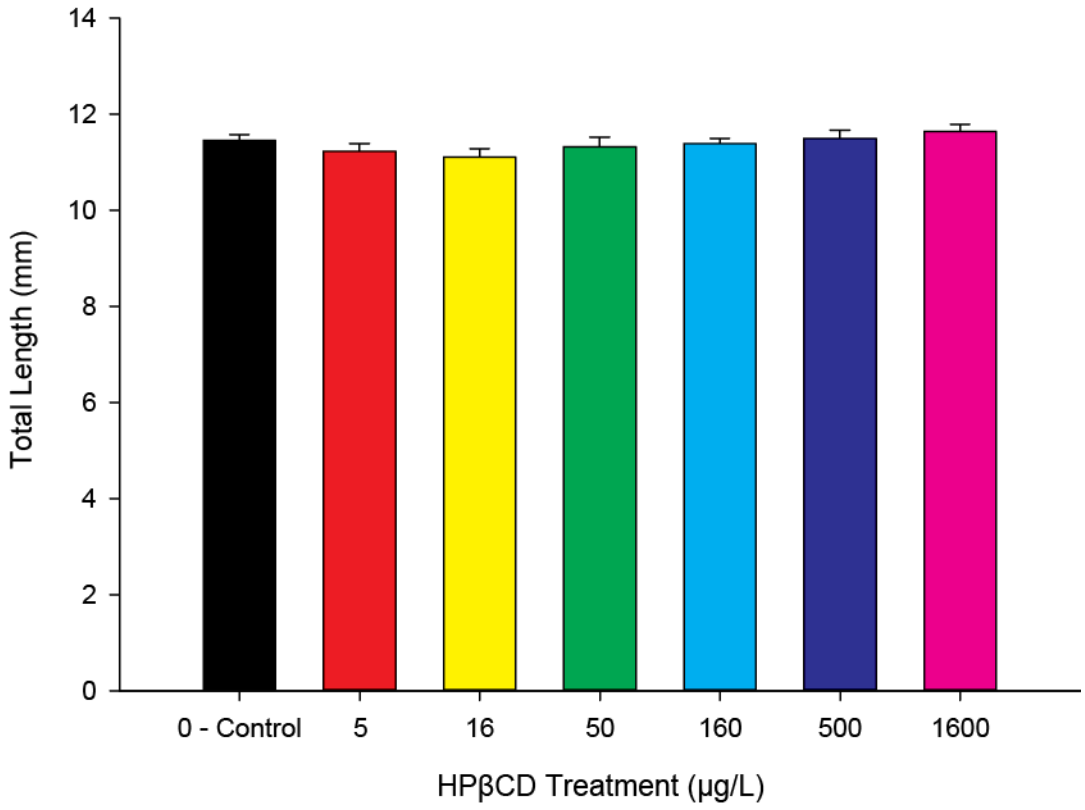

Figure 5: Total Length (mm) of 28 day old flagfish exposed to varying concentrations of HPβCD. Replicate data were pooled and reported as means ± SE. There was no statistically significant difference between treatments ($P \leq 0.05$). n= 44-55.

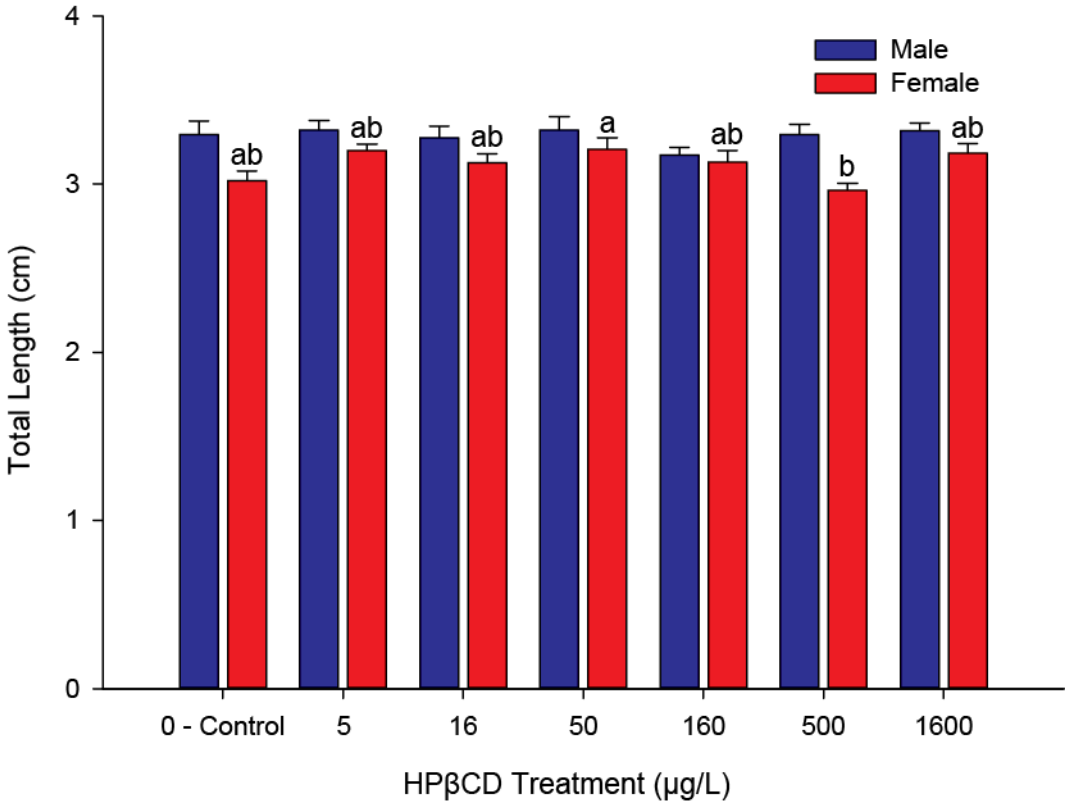

Figure 6: Total Length (cm) of 74-77 day old flagfish exposed to varying concentrations of HPβCD. Data were separated by sex and the results of the two replicates were pooled and reported as means ± SE. Within sex, bars without a letter in common are significantly different, no significant difference between males ($P \leq 0.05$). Male n=14-20, Female n=11-16.

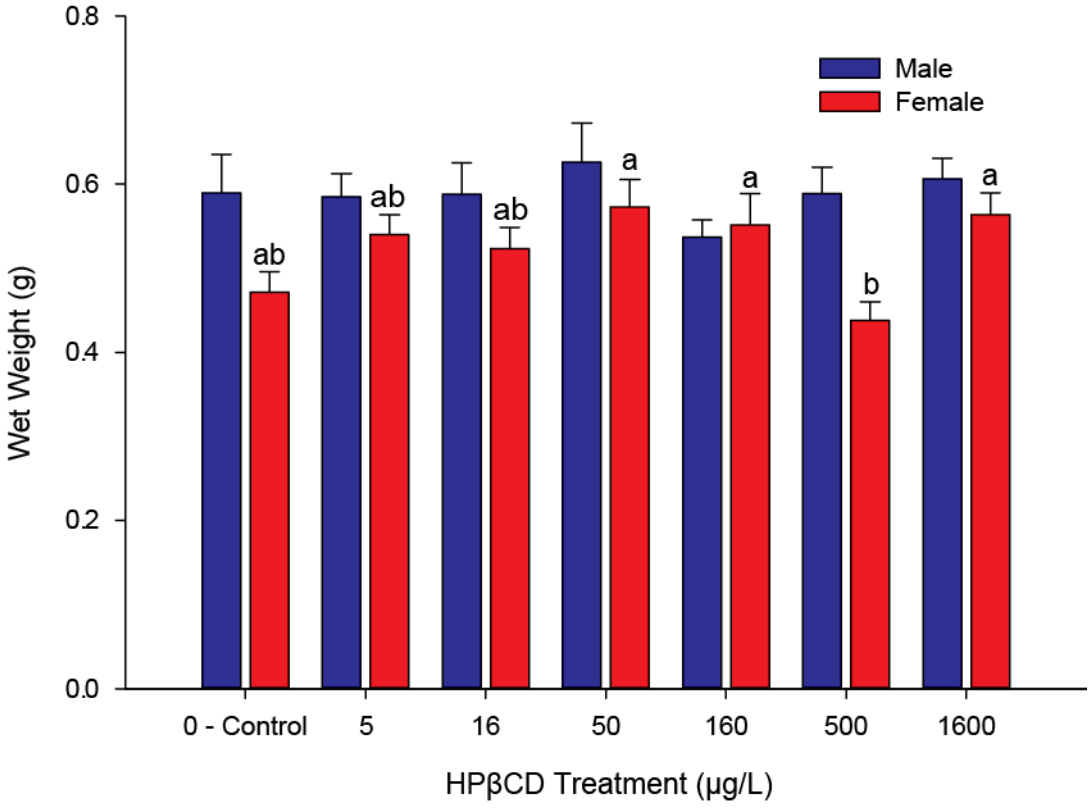

Figure 7: Wet weight (g) of 74-77 day old flagfish exposed to varying concentrations of HPβCD. Data were separated by sex and the results of the two replicates were pooled and reported as means ± SE. Within sex, bars without a letter in common are significantly different, no significant difference between males ($P \leq 0.05$). Male n=14-20, Female n=11-16.

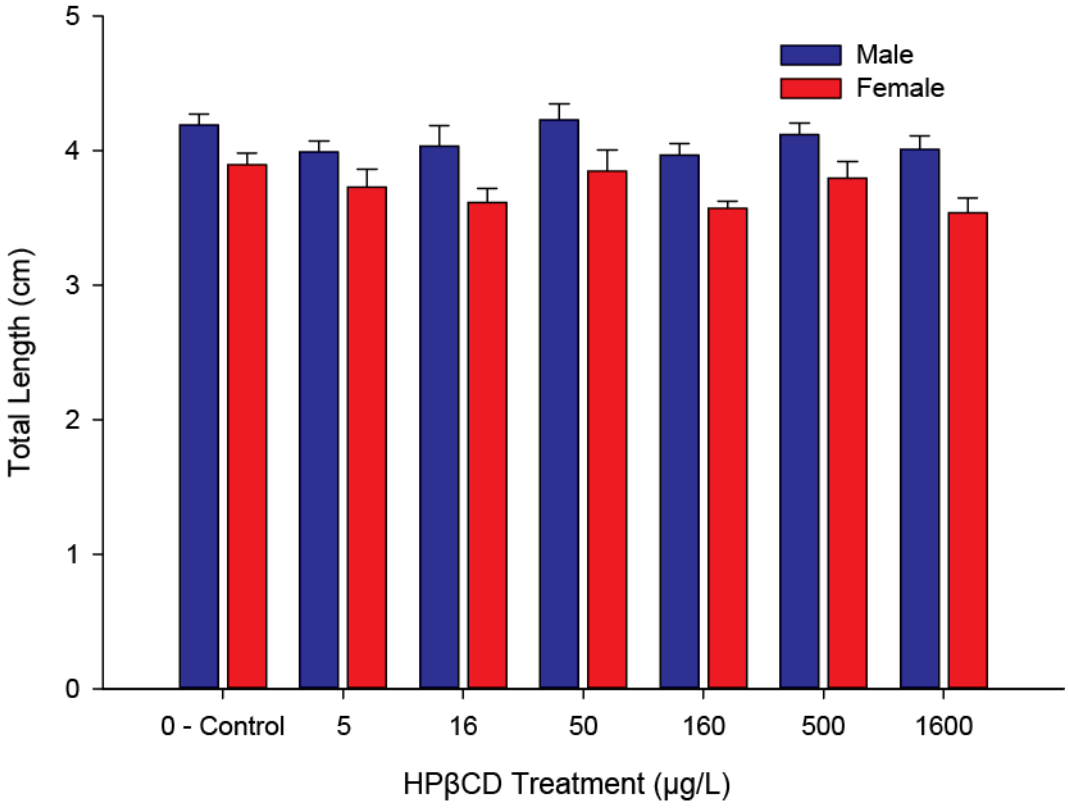

Figure 8: Total Length (cm) of 102-103 day old flagfish exposed to varying concentrations of HPβCD. Data were separated by sex and the results of the two replicates were pooled and reported as means ± SE. There was no statistically significant difference within sex ($P \leq 0.05$). Male n=7-13, Female n=4-9.

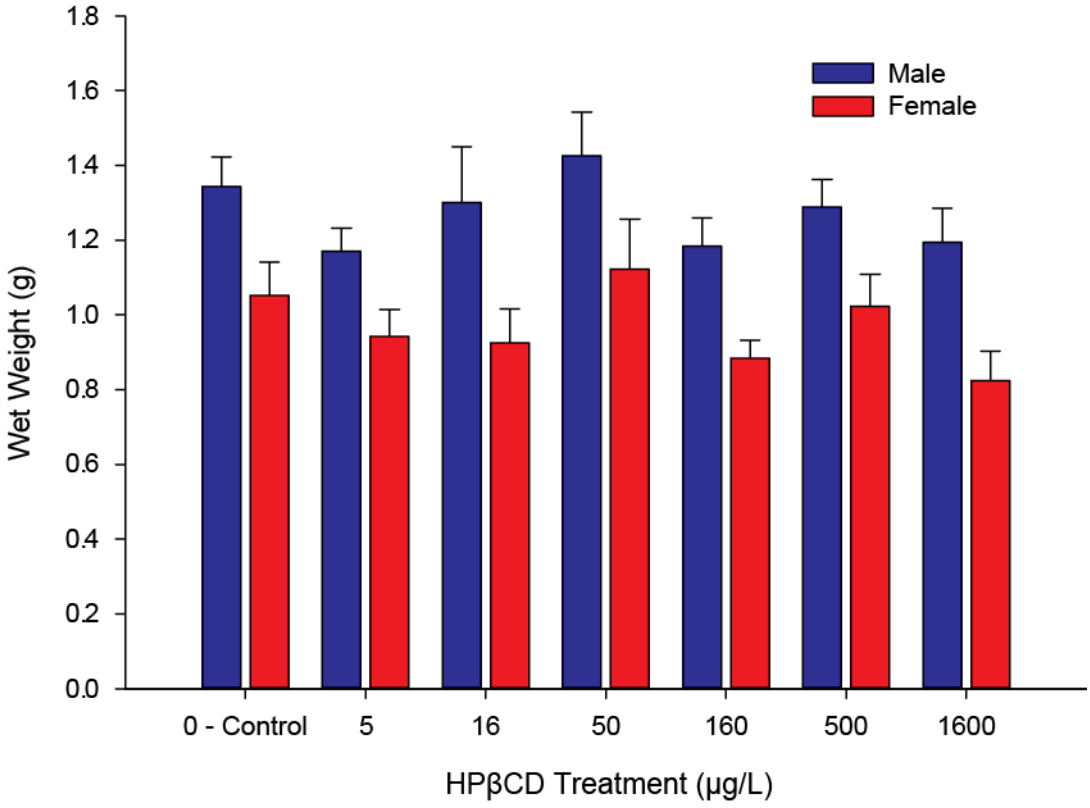

Figure 9: Wet weight (g) of 102-103 day old flagfish exposed to varying concentrations of HPβCD. Data were separated by sex and the results of the two replicates were pooled and reported as means ± SE. There was no statistically significant difference within sex ($P \leq 0.05$). Male n=7-13, Female n=4-9.

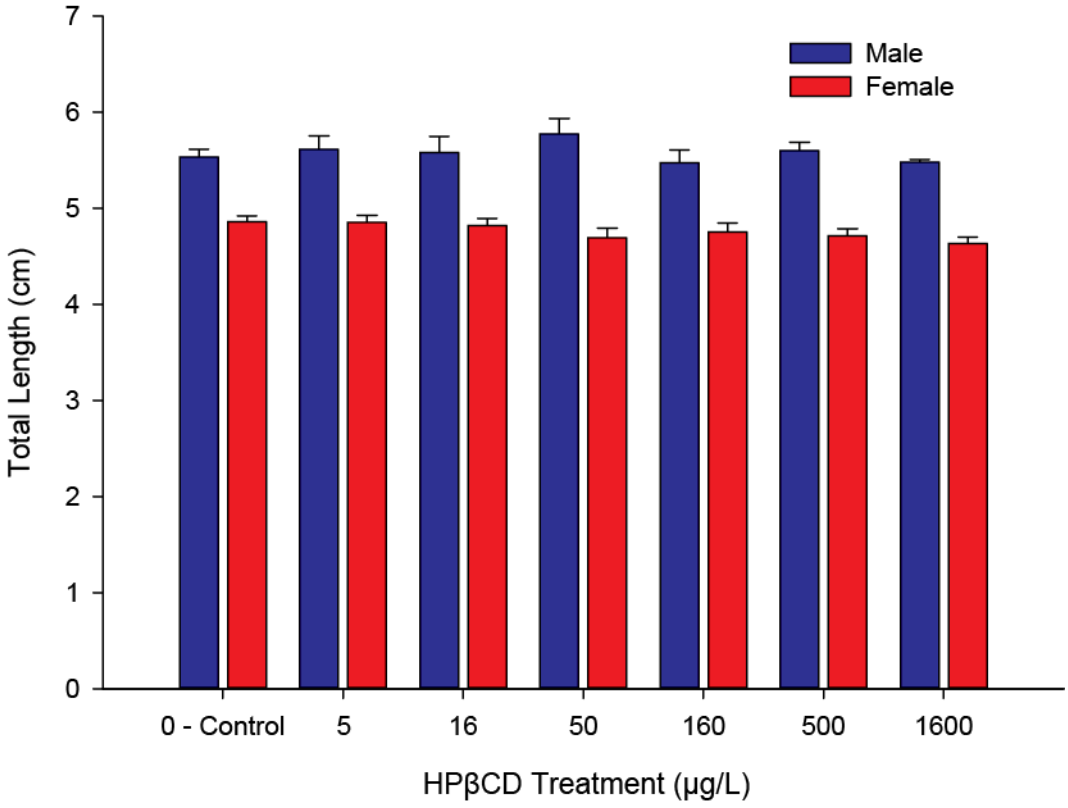

Figure 10: Total Length (cm) of 144-145 day old flagfish exposed to varying concentrations of HPβCD. Data were separated by sex and the results of the two replicates were pooled and reported as means ± SE. There was no statistically significant difference within sex ($P \leq 0.05$). Male n=4, Female n=8.

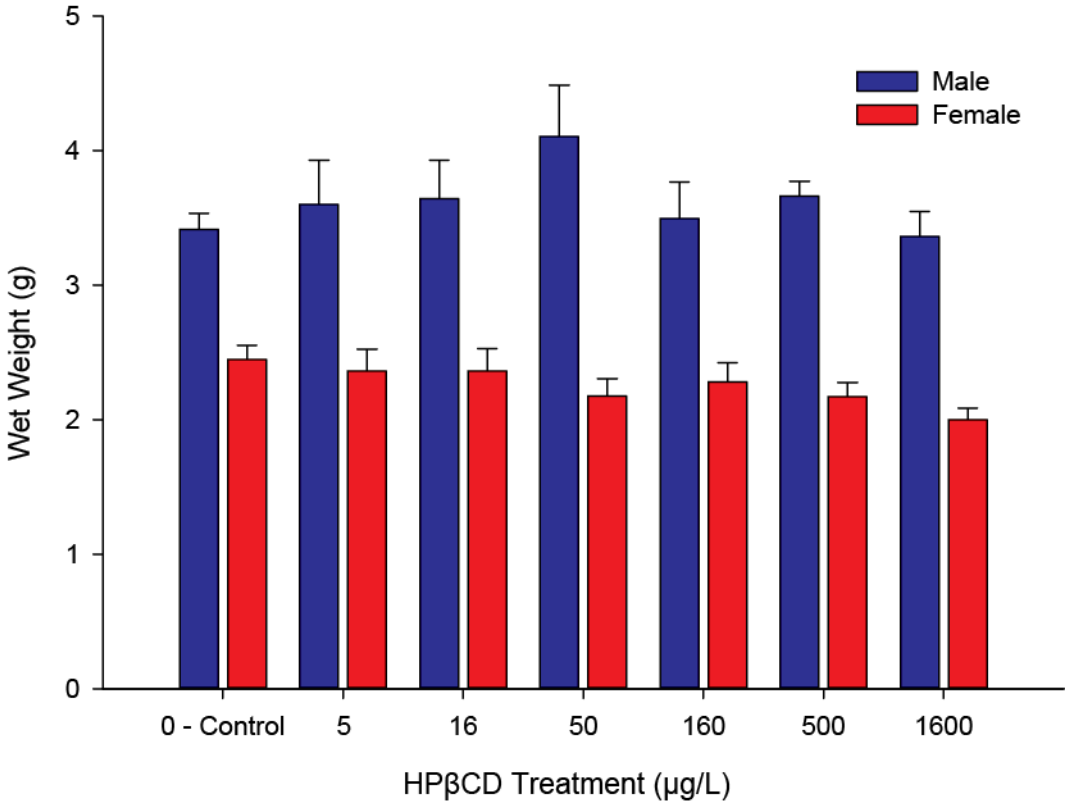

Figure 11: Wet weight (g) of 144-145 day old flagfish exposed to varying concentrations of HPβCD. Data were separated by sex and the results of the two replicates were pooled and reported as means ± SE. There was no statistically significant difference within sex ($P \leq 0.05$). Male n=4, Female n=8.

4.2.4 Condition Indices

4.2.4.1 Condition Factor

There were no significant differences in condition factor between replicates at 77-74 DPH, 102-103 DPH, and 144-145 DPH, thus, data was pooled. Data were analyzed based on gender. No significant differences were found between HPβCD treatments and the control in both males and females at 74-77, 102-103, or 144-145 DPH ($P \leq 0.05$) (Figures 11, 12, and 13).

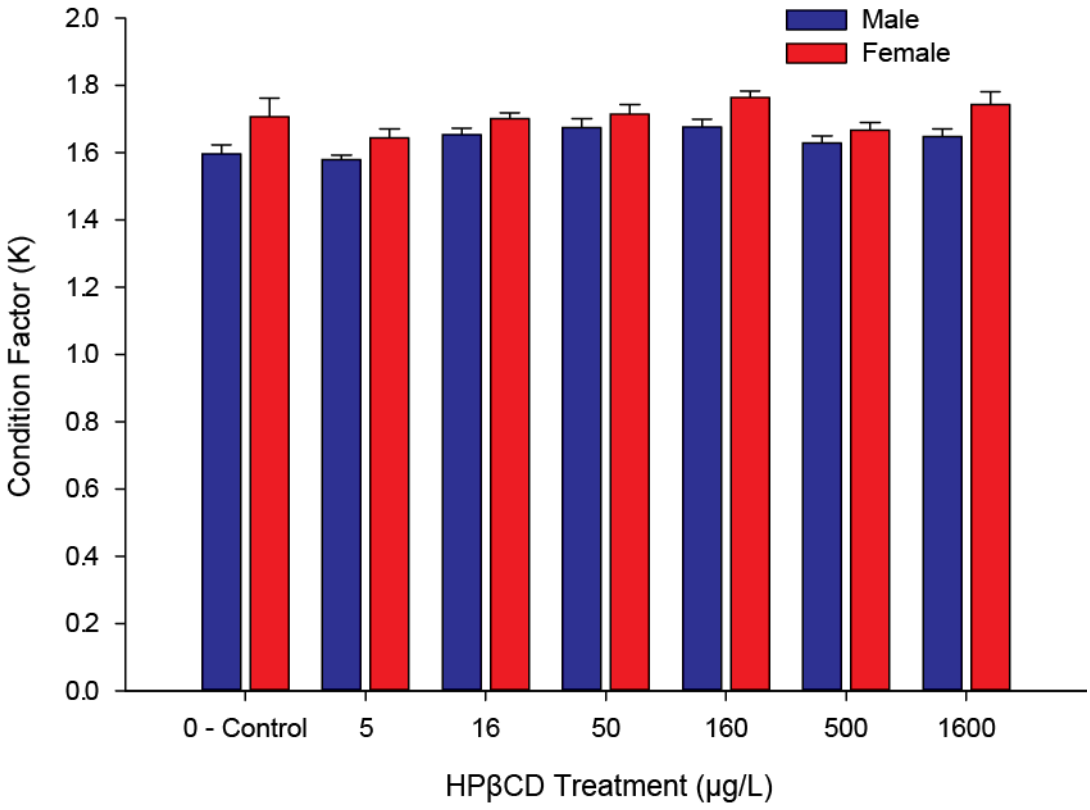

Figure 12: Condition Factor (K) of 74-77 day old flagfish exposed to varying concentrations of HPβCD. Data were separated by sex and the results of the two replicates were pooled and reported as means ± SE. There was no statistically significant difference within sex ($P \leq 0.05$). Male n=14-20, Female n=11-16.

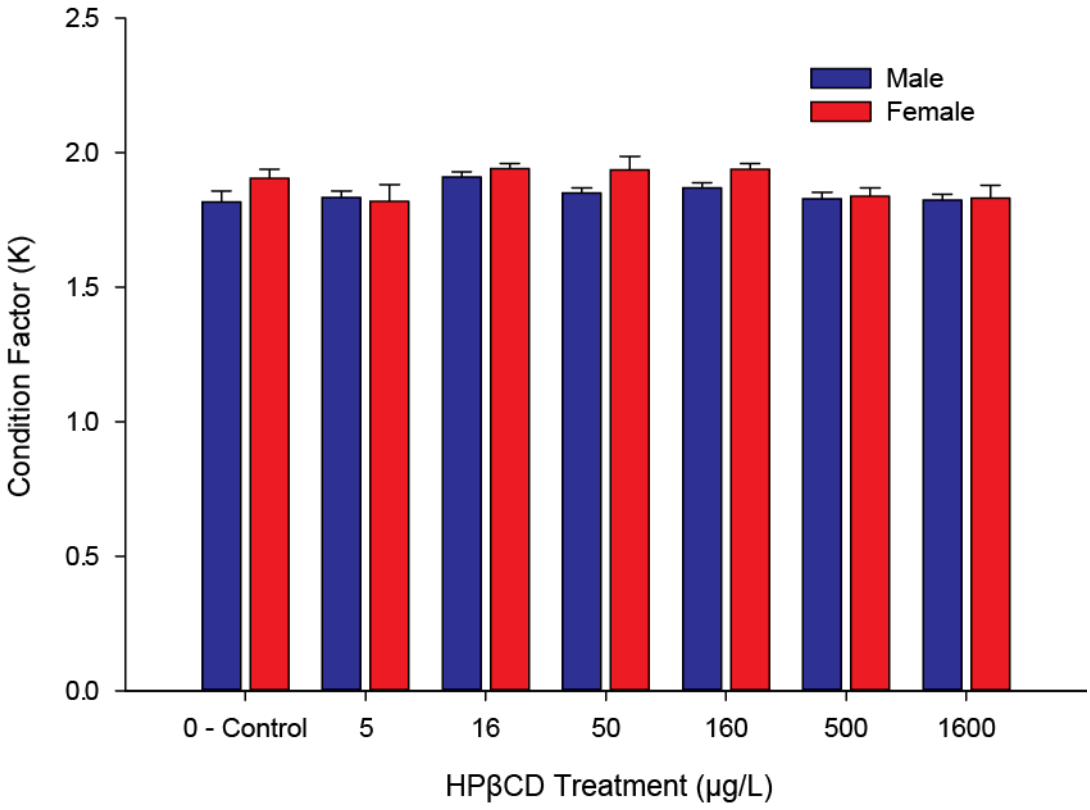

Figure 13: Condition Factor (K) of 102-103 day old flagfish exposed to varying concentrations of HPβCD. Data were separated by sex and the results of the two replicates were pooled and reported as means ± SE. There was no statistically significant difference within sex ($P \leq 0.05$). Male n=7-13, Female n=4-9.

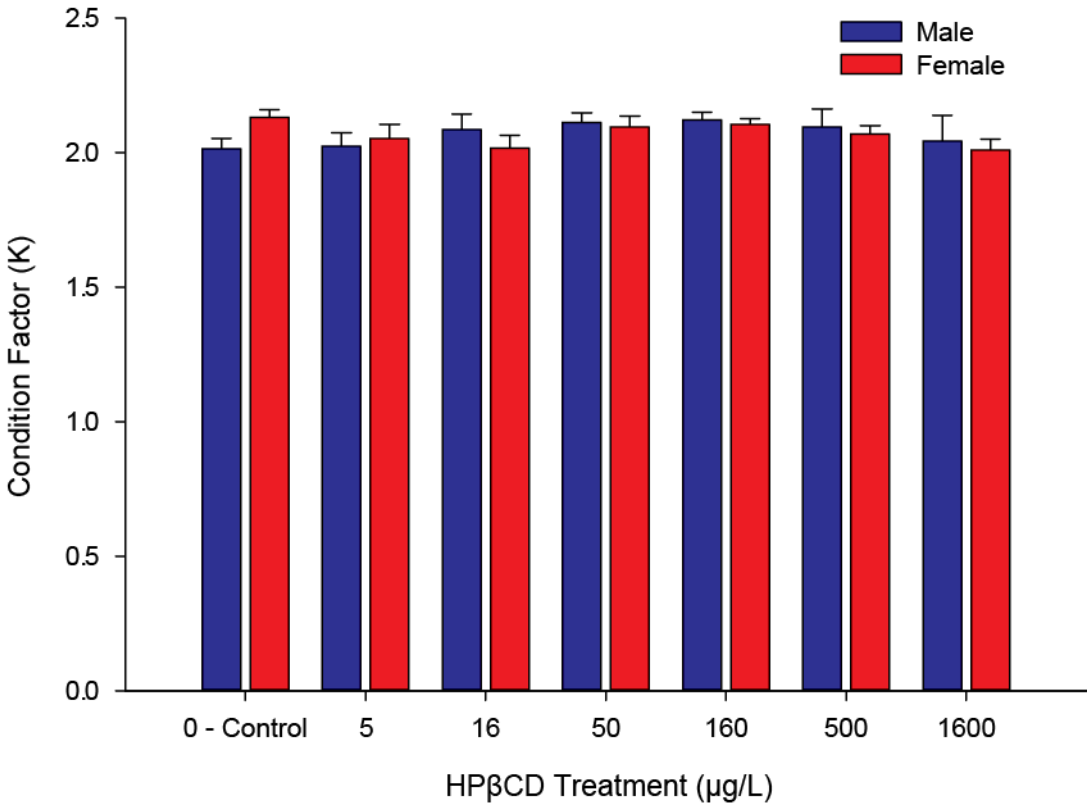

Figure 14: Condition Factor (K) of 144-145 day old flagfish exposed to varying concentrations of HPβCD. Data were separated by sex and the results of the two replicates were pooled and reported as means ± SE. There was no statistically significant difference within sex ($P \leq 0.05$). Male n=4, Female n=8.

4.2.4.2 Hepatosomatic Index (HSI)

There were no significant differences in HSI between replicates at 74-77, 102-103 or 144-145 DPH, and thus the data were pooled and re-analyzed ($P \leq 0.05$). No significant differences were observed in both males and females at 74-77, 102-103 or 144-145 DPH ($P \leq 0.05$) (Figures 14, 15, and 16).

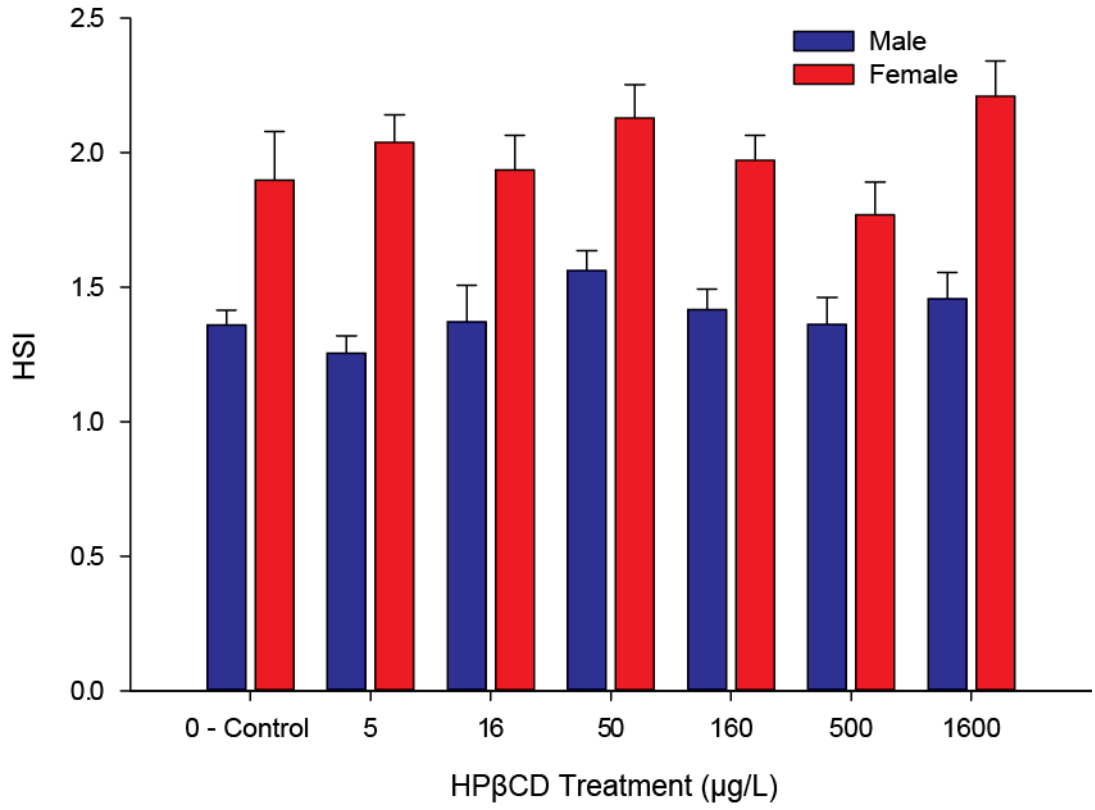

Figure 15: Hepatosomatic (HSI) of 74-77 day old flagfish exposed to varying concentrations of HPβCD. Data were separated by sex and the results of the two replicates were pooled and reported as means ± SE. There was no statistically significant difference within sex ($P \leq 0.05$). Male n=14-20, Female n=11-16.

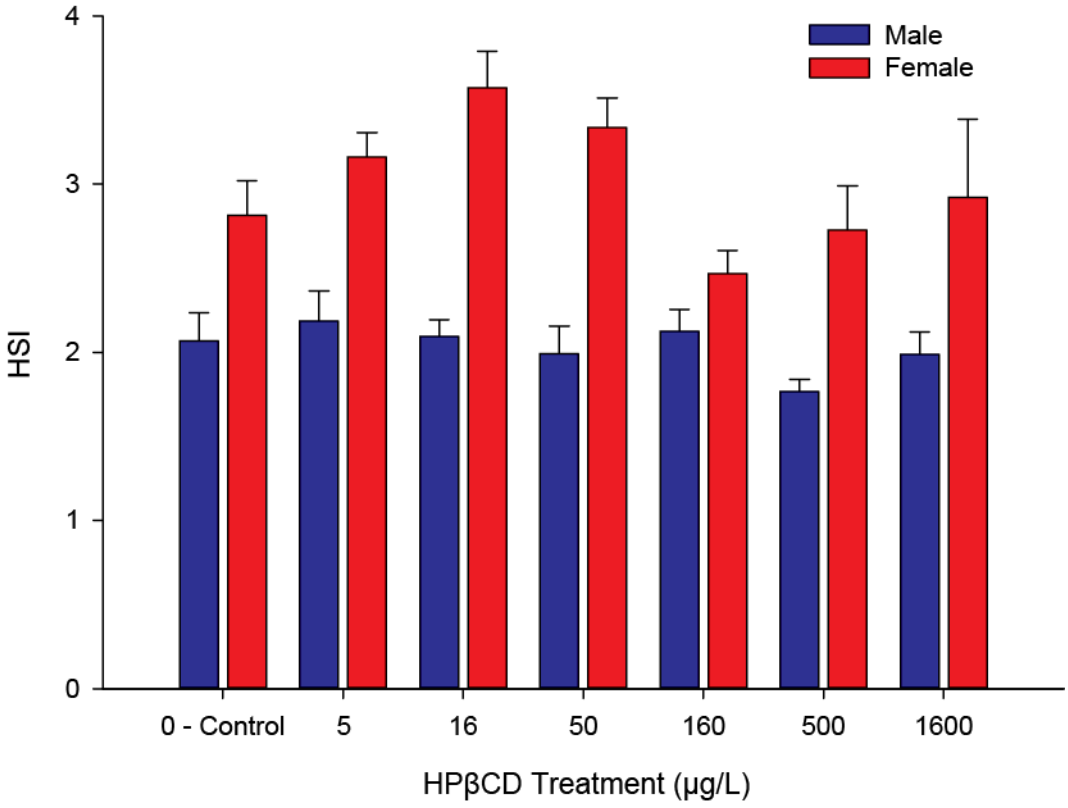

Figure 16: Hepatosomatic (HSI) of 102-103 day old flagfish exposed to varying concentrations of HPβCD. Data were separated by sex and the results of the two replicates were pooled and reported as means ± SE. There was no statistically significant difference within sex ($P \leq 0.05$). Male n=7-13, Female n=4-9.

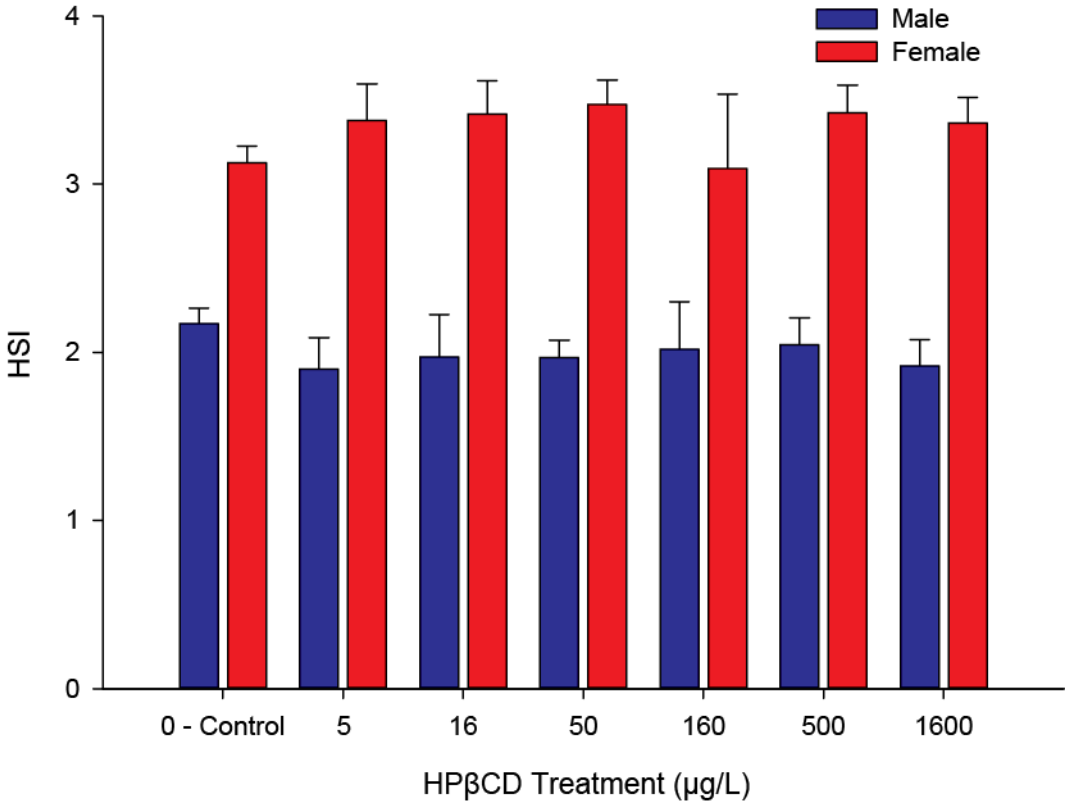

Figure 17: Hepatosomatic (HSI) of 144-145 day old flagfish exposed to varying concentrations of HPβCD. Data were separated by sex and the results of the two replicates were pooled and reported as means ± SE. There was no statistically significant difference within sex ($P \leq 0.05$). Male n=4, Female n=8.

4.2.4.3 Gonadosomatic Index (GSI)

Gonad weight of flagfish at 102-103 DPH was not significantly different between replicates, and thus data were pooled and re-analyzed ($P \leq 0.05$). No significant differences were found in GSI, between HPβCD treatments and the control at 102-103 DPH for either males or females ($P \leq 0.05$) (Figure 17).

No significant differences in GSI were observed between replicates at 144-145 DPH for either males or females and therefore data were pooled and re-analyzed ($P \leq 0.05$). A significant increase in GSI was observed in female flagfish exposed to 50 µg/L HPβCD ($P \leq 0.05$). Moreover, GSI of 5 and 50 µg/L treatments were significantly higher than the controls when analyzed at $P \leq 0.10$, protecting against Type Two error. Also noted was a trend that all HPβCD treatments showed a higher GSI than the controls, although not significantly when looked at individually (Figure 18). However, when all HPβCD treatments were pooled and analyzed via a t-test, a significant increase compared to the controls was observed in female GSI ($P \leq 0.05$) (Figure 19). There were no significant differences observed between the HPβCD treated males and the controls ($P \leq 0.05$).

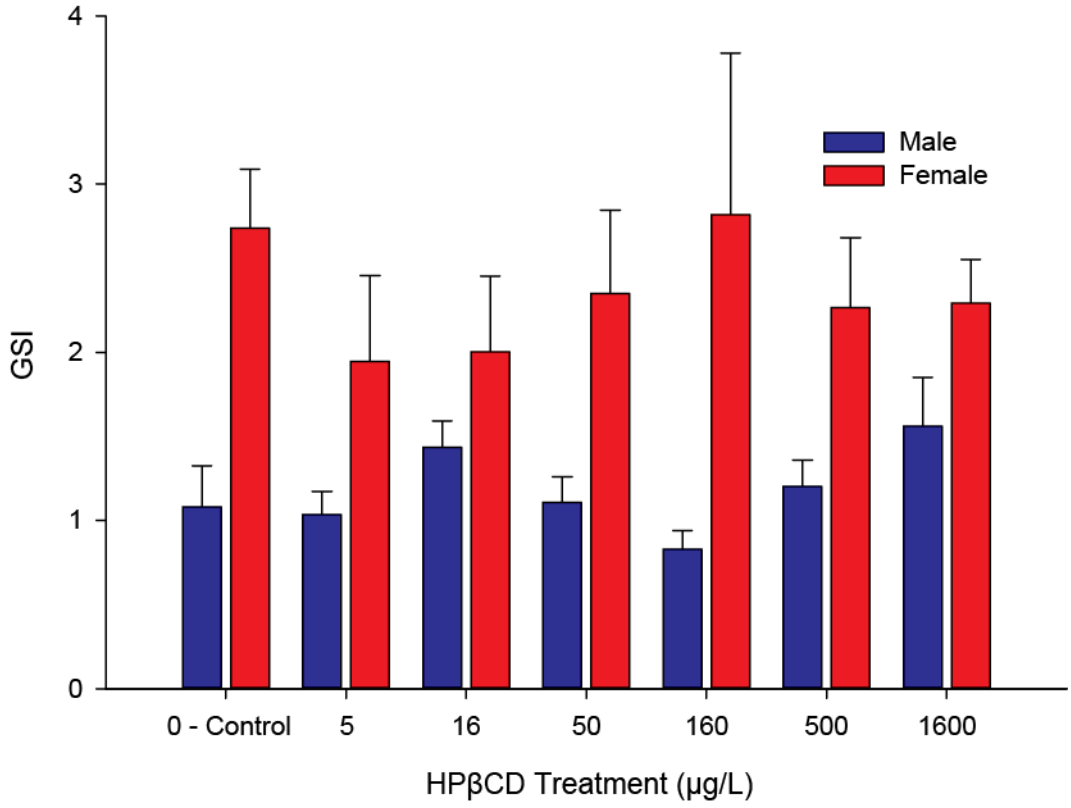

Figure 18: Gonadosomatic index (GSI) of 102-103 day old flagfish exposed to a variety of concentrations of HPβCD. Data were separated by sex and the results of the two replicates were pooled and reported as means ± SE. There was no statistically significant difference within sex ($P \leq 0.05$). Male n=4, Female n=8.

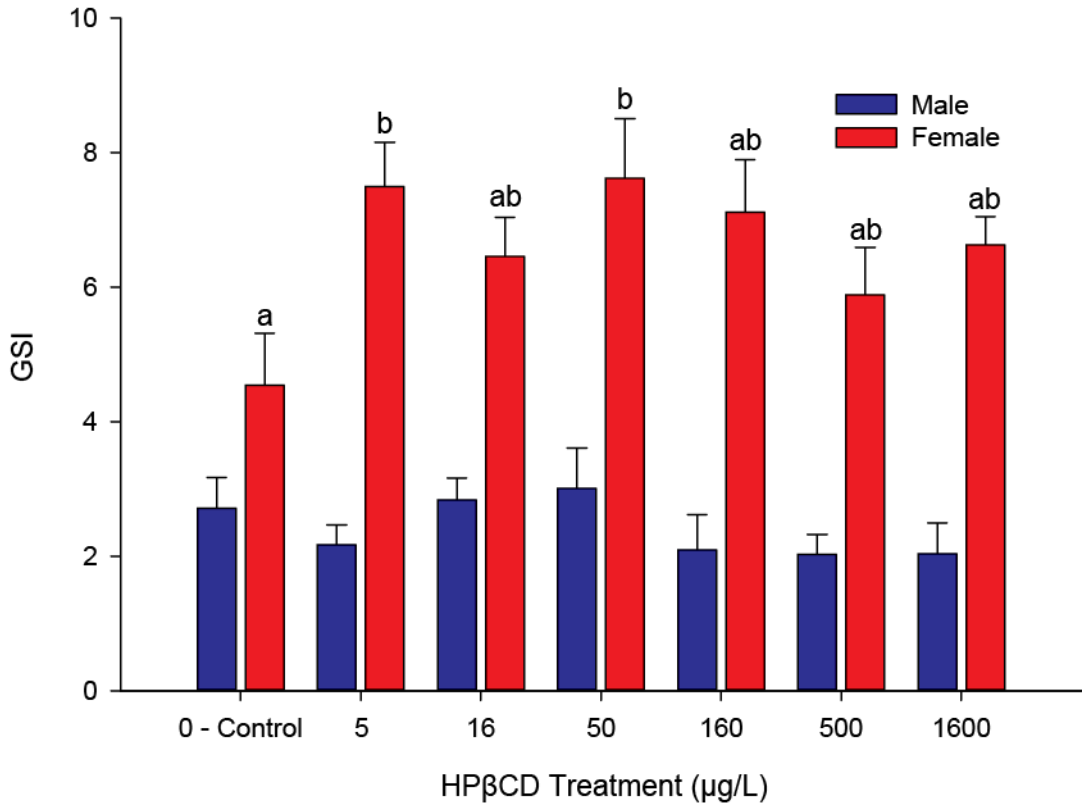

Figure 19: Gonadosomatic index (GSI) of 144-145 day old flagfish exposed to varying concentrations of HPβCD. Data were separated by sex and the results of the two replicates were pooled and reported as means ± SE. Within sex, bars without a letter in common are significantly different, no significant difference between males ($P \leq 0.10$). Male n=4, Female n=8.

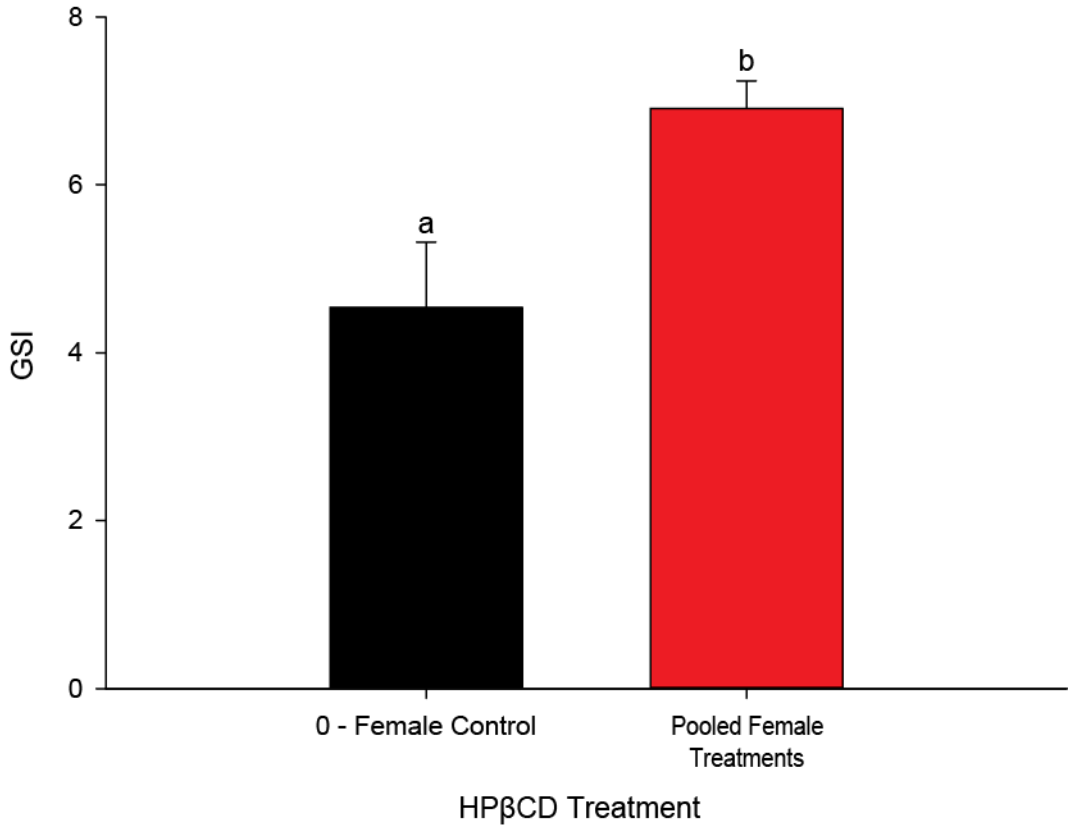

Figure 20: Gonadosomatic index (GSI) of 144-145 day old female flagfish exposed to varying concentrations of HPβCD. HPβCD treatments were not statistically different from one another and were pooled, means ± SE were compared to the control. Bars without a letter in common are significantly different ($P \leq 0.05$). Control n=8, HPβCD treatment n=48.

4.2.5 Reproductive Output

Reproductive activity was monitored for 28 days during the breeding period. It was found that the replicates were not significantly different from one another and therefore were pooled for analysis. No HPβCD treatment had a significant impact on the time to first egg laying ($P \leq 0.05$) (Table 4). The overall mean time to first egg laying was 110 ± 0.5 DPH.

Time to steady state spawning, defined as 30 or more eggs laid 4 or more days in a row, was significantly shorter in fish exposed to the 1600 µg/L HPβCD treatment, with a mean time of 109 ± 0.0 DPH compared to the controls with a mean time of 126 ± 3.5 DPH ($P \leq 0.05$) (Table 4). There were no other significant differences between any other HPβCD treatments and the controls.

The number of days eggs were laid in HPβCD treatments of 16, 160, and 1600 µg/L were significantly higher than the controls at 20 ± 2 days ($P \leq 0.05$) (Table 4). This trend was not observed in the other HPβCD treatments. For example, the 500 µg/L HPβCD treatments experienced the fewest number of days in which eggs were laid with 17 ± 5 days.

The total number of eggs produced and the mean daily egg production were not significantly different between the HPβCD treatments and the controls ($P \leq 0.05$) (Table 4). However, it was found that the 1600 µg/L HPβCD treatment produced the greatest total number of eggs with 8430 ± 1360 eggs, and the highest mean daily egg production with 300 ± 48 eggs per day. On the other hand, the 500 µg/L treatment produced the fewest total eggs with 1170 ± 102 eggs, and the lowest mean daily egg production of 69 ±

21 eggs per day. For comparison, the controls produced 2970 ± 1460 eggs in total and a mean daily egg production of 150 ± 75 eggs per day (Table 4).

Table 4: The impact of chronic exposure to varying concentrations of HPβCD over a complete life-cycle on the reproductive ability of the American flagfish. Values are given as means ± SE. Results of the two replicates were pooled. Within columns, values without a letter in common are significantly different ($P \leq 0.05$).

HPβCD Treatment (µg/L)	Time to First Egg Laying (d)	Time to Steady State Spawning (d)	Number of Days Eggs Laid (d)	Total Number of Eggs Produced	Mean Daily Egg Production
0 – Control	112 ± 3.0	126 ± 3.5^{ab}	20 ± 2.0^{a}	2970 ± 1460	150 ± 75
5	110 ± 1.0	125 ± 6.0^{ab}	23 ± 3.0^{ab}	1760 ± 1290	77 ± 57
16	109.5 ± 0.5	113 ± 2.0^{ab}	27.5 ± 0.5^{b}	5400 ± 787	197 ± 29
50	110.5 ± 1.0	117 ± 7.5^{ab}	25.5 ± 2.5^{ab}	3870 ± 2540	152 ± 100
160	109.5 ± 0.5	114 ± 3.0^{ab}	27.0 ± 0^{bc}	4810 ± 430	178 ± 16
500	110.5 ± 1.5	130 ± 0.5^{a}	17.0 ± 5.0^{abc}	1170 ± 102	69 ± 21
1600	109 ± 0	109 ± 0^{b}	28.0 ± 0^{bc}	8430 ± 1360	301 ± 48
Average	**110.1 ± 0.1**	**-**	**-**	**4050 ± 1210**	**105 ± 50**

4.2.6 Reproductive Viability

Reproductive viability was monitored for 28 days during the breeding period. Percent fertilization, percent hatch, time to hatch, and percent malformed were all analyzed. With regards to percent fertilization, all HPβCD treatments, with the exception of the 5 µg/L HPβCD treatment, were not significantly different from the controls ($P \leq 0.05$). The controls had an average percent fertilization of 85.0 ± 2.1 %, while the 5 µg/L HPβCD treatment was significantly lower with a mean percent fertilization of 71.9 ± 3.2 % (Table 5 and Figure 20).

Additionally, there were no significant differences in percent hatch of fertilized eggs amongst HPβCD treatments when compared to the controls ($P \leq 0.05$) (Table 5). An overall experimental mean percent hatch of 99.0 ± 0.1 % was achieved during the breeding period. Time to hatch was also not significantly altered by any HPβCD treatment when compared to the controls ($P \leq 0.05$) (Table 5). The overall mean time to hatch was 5.2 ± 0.02 days.

The percent of malformed larval flagfish was significantly higher in the 160 µg/L HPβCD treatment when compared to the controls ($P \leq 0.05$) (Table 5 and Figure 21). The controls had a mean percent malformation of 0.67 ± 0.19 %, while the 160 µg/L HPβCD treatments had a mean percent malformation of 6.69 ± 1.09 %. An increase in larval malformation was not observed in any other HPβCD treatment when compared to the controls. It is important to note that the increased incidence of malformation in the 160 µg/L of HPβCD treatments only occurred between days 12 and 21 of the 30 day breeding period.

Table 5: The impact of chronic exposure to HPβCD over a complete life-cycle on egg viability of flagfish. Values are given as means ± SE. Results of the two replicates were pooled. Within columns, values without a letter in common are significantly different ($P \leq 0.05$).

HPβCD Treatment (µg/L)	% Fertilization	% Hatch	Time to Hatch (Days)	% Malformed
0 – Control	85.1 ± 2.1[a]	99.8 ± 0.1	5.4 ± 0.08	0.7 ± 0.2[a]
5	71.9 ± 3.2[b]	97.8 ± 1.0	5.3 ± 0.07	1.2 ± 0.4[a]
16	85.5 ± 1.4[a]	99.2 ± 0.7	5.1 ± 0.04	0.3 ± 0.1[a]
50	83.4 ± 2.1[ab]	99.2 ± 0.8	5.2 ± 0.07	0.6 ± 0.3[a]
160	81.2 ± 2.6[ab]	97.9 ± 1.1	5.2 ± 0.06	6.7 ± 1.1[b]
500	87.5 ± 2.0[a]	99.7 ± 0.2	5.2 ± 0.07	2.5 ± 0.9[a]
1600	86.6 ± 1.0[a]	99.9 ± 0.1	5.1 ± 0.05	0.3 ± 0.1[a]
Average	**-**	**99.0 ± 0.1**	**5.2 ± 0.02**	**-**

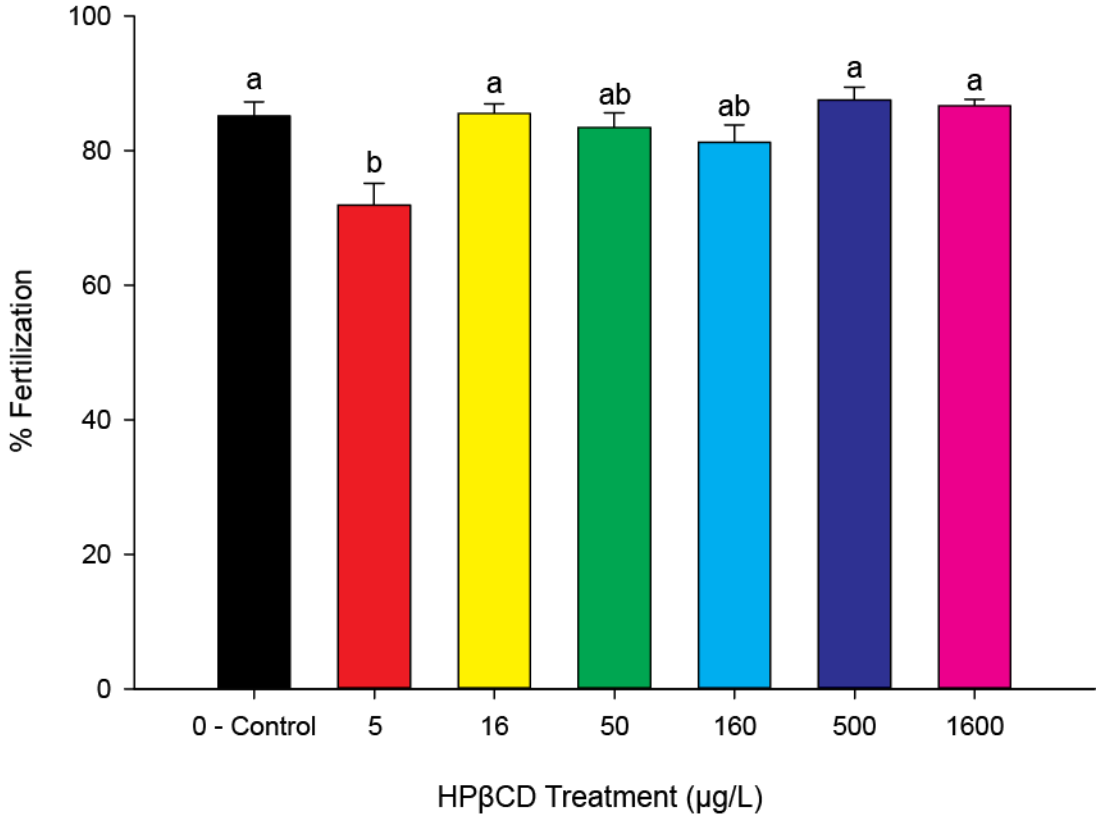

Figure 21: The impact of chronic exposure to HPβCD over a complete life-cycle on the percent fertilization of flagfish eggs. Values are given as means ± SE. Results of the two replicates were pooled. Within columns, values without a letter in common are significantly different ($P \leq 0.05$).

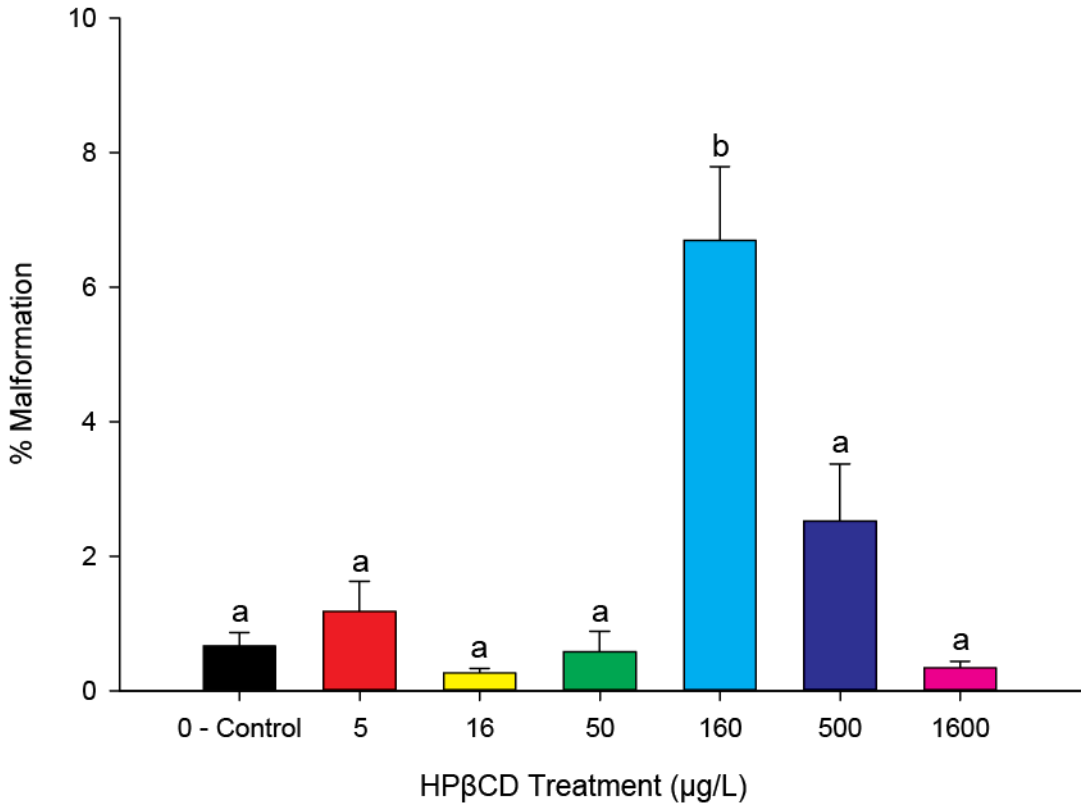

Figure 22: The impact of chronic exposure to HPβCD over a complete life-cycle on the percent malformation of larval flagfish. Values are given as means ± SE. Results of the two replicates were pooled. Within columns, values without a letter in common are significantly different ($P \leq 0.05$).

4.2.7 Second Generation Survival and Growth

Survival and length of second generation juvenile flagfish reared in the same HPβCD treatments as their parents were monitored over 28 days. Very little mortality was observed during the 28 days, and the HPβCD treatments were not statistically different from the controls ($P \leq 0.05$) (Table 6).

Total length was measured at 21 DPH; replicates were pooled and reported as means ± SE (Figure 22). Second generation 21 DPH American flagfish exposed to 5, 16, 50, 160 and 500 µg/L HPβCD were smaller in length when compared to the controls ($P \leq 0.05$) while fish exposed to 1600 µg/L HPβCD were not statistically different from the controls.

At 28 DPH a significant difference was observed between the lengths of the controls and the 500 µg/L HPβCD treatment ($P \leq 0.05$) (Figure 23). All other HPβCD treatments were not statistically different from the controls.

Table 6: The impact of chronic exposure to HPβCD over a complete life-cycle on the survival of second generation American flagfish. Two replicates were pooled for each treatment (n=60) and values are given as means ± SE. There was no statistically significant difference between treatments ($P \leq 0.05$).

HPβCD Treatment (µg/L)	**n**	**% Mortality**
0 – Control	60	1.6 ± 1.6
5	60	1.6 ± 1.6
16	60	3.3 ± 3.3
50	60	3.3 ± 0.0
160	60	3.3 ± 3.3
500	60	3.3 ± 0.0
1600	60	3.3 ± 3.3

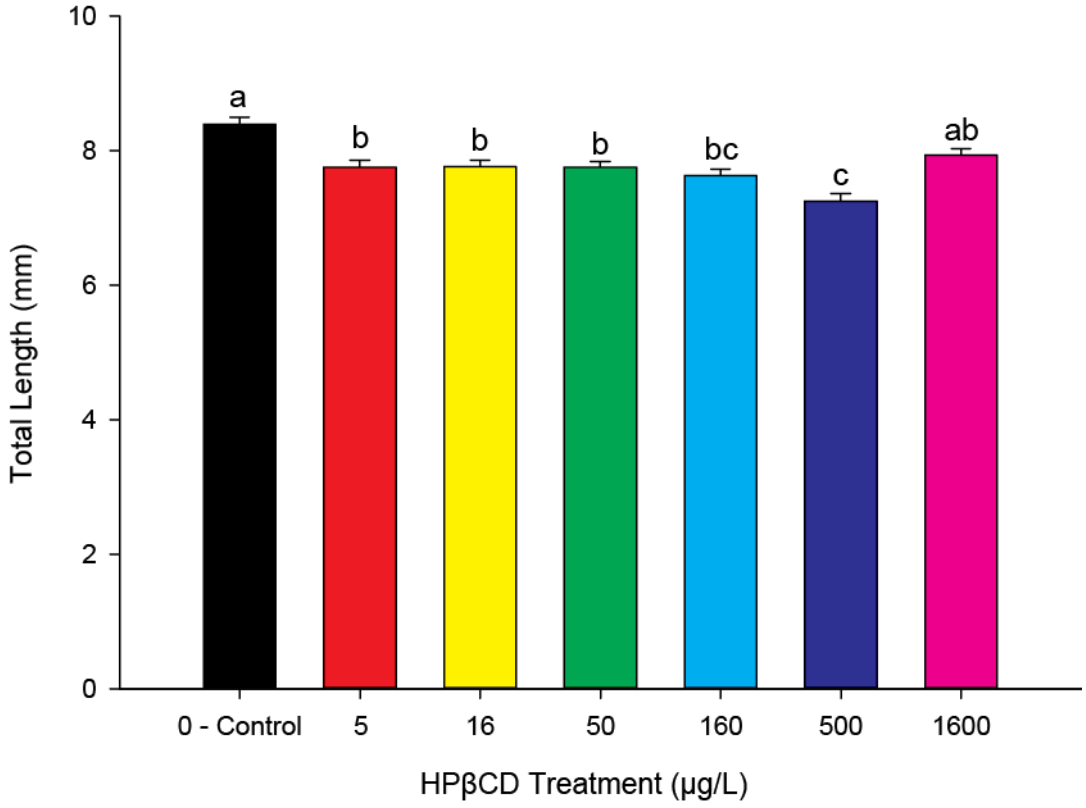

Figure 23: Total Length (mm) of 21 day old second generation flagfish from a full life-cycle of exposure to varying concentrations of HPβCD. Replicates were pooled and reported as means ± SE. Bars without a letter in common are significantly different ($P \leq 0.05$). n=54- 60.

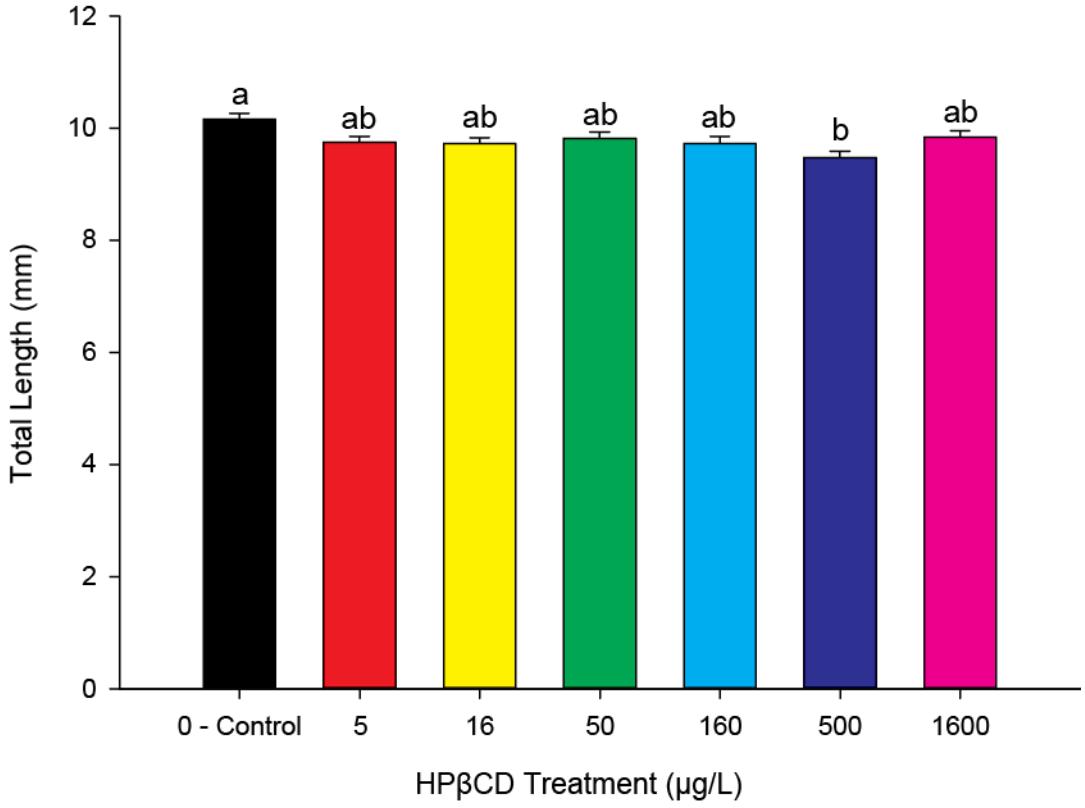

Figure 24: Total Length (mm) of 28 day old second generation flagfish from a full life-cycle of exposure to varying concentrations of HPβCD. Replicates were pooled and reported as means ± SE. Bars without a letter in common are significantly different ($P \leq 0.05$). n=43-53.

4.2.8 96-h Copper Toxicity Challenge

Acute toxicity of copper was determined for the larval offspring of parents chronically exposed to HPβCD for a full life-cycle; cumulative percent mortality over 96-h was calculated and plotted as a contour graph (Figure 24). Trends in offspring sensitivity to copper were determined by changes in cumulative mortality at given copper concentrations. Offspring from parents exposed to HPβCD were moderately more sensitive to copper. It should be noted that at 1600 µg/L HPβCD this sensitivity appears to diminish at high concentrations of copper.

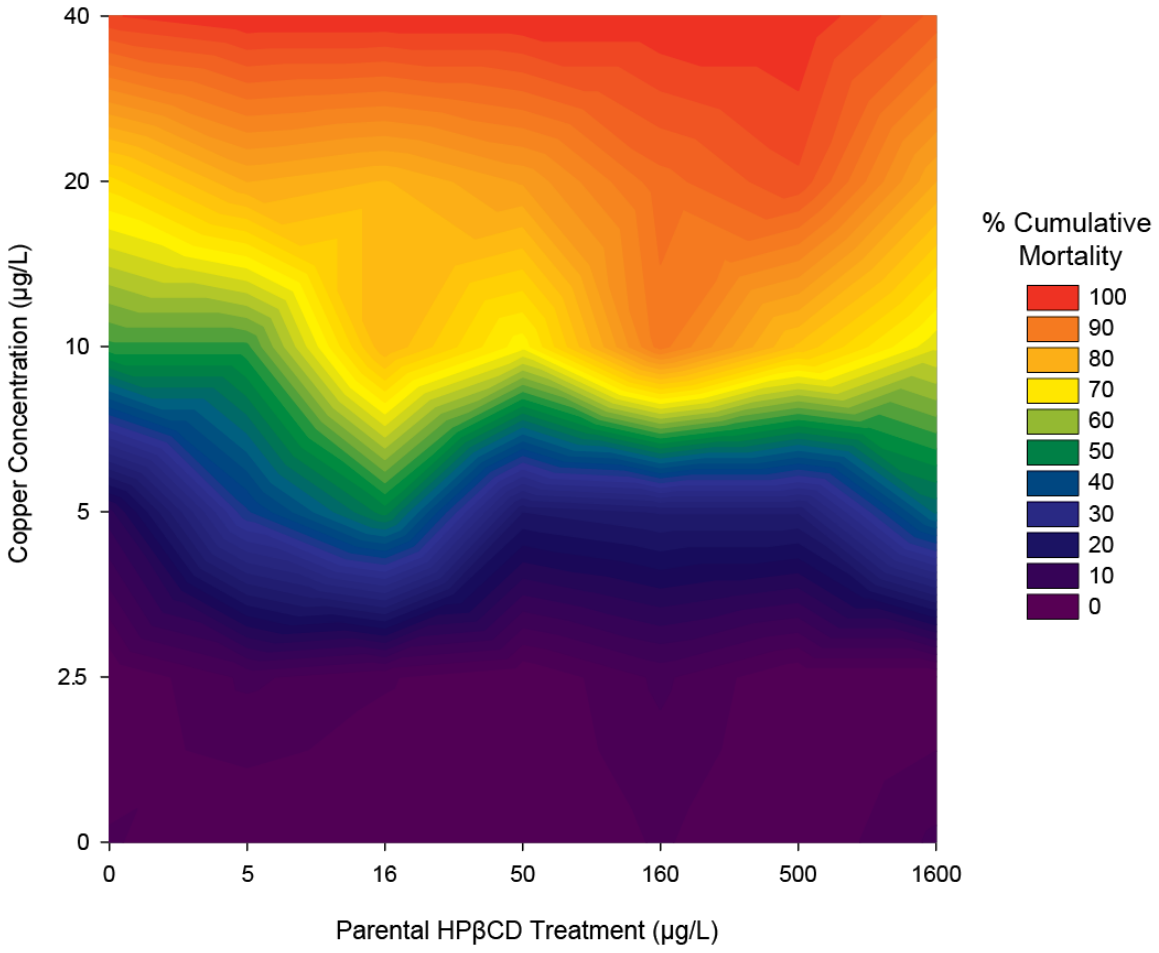

Figure 25: Mortality from a 96-h copper exposure to American flagfish larval offspring from parents chronically exposed to HPβCD. Replicates were pooled and reported as mean percent cumulative mortality. n=45 flagfish for each parental treatment at each copper concentration.

5.0 Discussion

Pharmaceuticals and personal care products are ever-growing contaminants in both water systems and the environment due to their extensive uses and poor disposal methods. During this investigation, HPβCD, a common PPCP, was investigated both acutely and chronically on the American flagfish.

5.1 Acute Larval Toxicity of HPβCD

Understanding the acute toxicity of HPβCD is important for many reasons. Acute toxicity often indicates the immediate impacts on the survival of the exposed organisms as well as how to manage environmental contamination based on the relative toxicity of the contaminant. Acute toxicity in aquatic models is often defined as the concentration at which 50% cumulative mortality is observed over a 96 hour exposure (Sprague, 1969). Little to no mortality was observed in larval American flagfish exposed to concentrations up to 1 g/L of HPβCD for 96 hours. These results indicate that HPβCD is not acutely toxic to larval American flagfish. This finding is consistent with studies completed on mammalian models where doses of HPβCD up to 4500 mg/kg/day produced no significant impact on the health of rats over a 14 day exposure (Gould and Scott, 2005). Previously, no information regarding the acute toxicity of HPβCD to fish as a non-target organism was available.

This acute toxicity study of HPβCD is important as it shows that HPβCD is not acutely toxic to larval flagfish. The concentrations tested were well above any expected environmental concentrations of HPβCD (Rosi-Marshall and Royer, 2012). Moreover, the lack of acute toxicity of HPβCD to larval American flagfish provided reassurance that

chronic exposure over a complete life-cycle would allow desired endpoints to be monitored without being confounded by mortality.

5.2 Chronic Toxicity of HPβCD

5.2.1 Chronic Mortality Effects of HPβCD

No significant mortality of American flagfish was observed over the course of a full life-cycle exposure of up to 1600 µg/L HPβCD (Table 3). This is consistent with other studies that examined the effects of HPβCD on the survival of various mammalian models including rats, mice, and monkeys (Gould and Scott, 2005). However, this is the first study completed on an aquatic organism, and indicates that chronic exposure of American flagfish to HPβCD over one complete life-cycle has little impact on overall survival.

5.2.2 Chronic Growth Effects of HPβCD

Monitoring growth of fish is a simple, but effective method of assessing general energetic effects that a toxicant may be imposing. A difference in either length or weight may indicate an alteration in energy investments may be occurring. It has been reported that growth is not the most reliable method for determining fish bioenergetics due to the strong influences that temperature, feed, and aquarium densities have on it (Kaushik, 1998). However, when examined in a laboratory setting, with most modifying factors controlled for, growth can be an important pre-screening tool. Increased energy allotment for processes such as detoxification may reduce the available energy for growth, resulting in a smaller size than organisms not similarly exposed (Sherwood *et al.*, 2000).

Alterations in the bioenergetics of fish can dramatically impact survivability in the wild (Sherwood *et al.,* 2000).

Very little variation in the total length and wet weight of all life-stages of American flagfish chronically exposed to HPβCD was observed (Figures 3, 4, 5, 6, 7, 8, 9, and 10). Any differences in growth were not observed in all HPβCD concentrations nor did they occur in a concentration-dependent manner. Although growth is a good initial bioenergetics endpoint in fish, many other factors can influence growth. Temperature, food availability, and fish density all have very large influences on fish growth (Sprague, 1971). Fish density was kept consistent between treatments over the course of the experiment by thinning at appropriate intervals. Temperature was also kept constant through the use of a temperature-control room as well as a flow-through dosing system. Although food was fed in a consistent manner to each aquarium, it was not explicitly rationed, and small variances in food availability may explain the slight discrepancies in growth. As a result, any growth effects were most likely due to alternative factors such as food availability.

5.2.3 Chronic Effects of HPβCD on Condition Indices

5.2.3.1 Effects of HPβCD on Condition Factor

Condition factor is a relationship between the length and weight of fish and is often used as an initial indicator of overall fish health (van der Oost, *et al.,* 2003). Changes in condition factor can be due to an alteration in overall fish health (van der Oost, *et al.,* 2003). In this study, chronic exposure to HPβCD had no impact on the condition factor of flagfish when compared to the controls (Figures 11, 12, and 13). Thus

no major health impact of chronic exposure of American flagfish to HPβCD was observed based on condition factor.

5.2.3.2 Effects of HPβCD on HSI

Calculating HSI is an effective preliminary tool for understanding the impacts of a toxicant on the liver because HSI is a ratio of liver weight to total body weight in fish (van der Oost, *et al.,* 2003; Sadekarpawar and Parikh, 2013). Since the liver is a major organ in detoxification, an increase in liver size can be indicative of toxicant exposure (van der Oost, *et al.,* 2003). Increasing liver weight can result from either hyperplasia, an increase in the number of cells in the liver, or from hypertrophy, an increase in the volume of the cells in the liver (van der Oost, *et al.,* 2003). Increases in HSI may be related to an amplified liver detoxification processes that often accompanies toxicant exposure (Whyte *et al.,* 2000; van der Oost, *et al.,* 2003).

HPβCD had no impact on HSI when compared to the controls at each time point investigated in this study. This suggests that HPβCD is probably not detoxified within the liver of the American flagfish (Figures 14, 15, and 16). This result is supported by mammalian studies which have shown little to no histopathological changes in rats when exposed to high doses (4500 mg/kg/day) of HPβCD (Gould and Scott, 2005). Further research, including histo-pathological studies as well as monitoring EROD (ethoxyresorufin-O-deethylase) activity would be required to confirm this finding in fish (Whyte *et al.,* 2000)

5.2.3.3 Effects of HPβCD on GSI

GSI is a ratio of gonad weight to total body weight in fish. Examining GSI provides an approximate assessment of reproductive maturity and permits a gross measure of toxicant impacts on the reproductive capabilities of a fish. Generally, a decrease in GSI is indicative of a reduction in reproductive abilities, whereas an increase in GSI may indicate reproductive stimulation (Brewer, *et al.*, 2007).

HPβCD had no effect on flagfish GSI at 102-103 DPH in both males and females (Figure 17). The large variability observed is most likely due to individual variability in reaching sexual maturity within treatments. However, on days 144-145 DPH following the 30 day breeding period, a significant increase in female GSI compared to the controls was observed in the 5 and 50 µg/L HPβCD treatments ($P \leq 0.10$) (Figure 18). Although the 50 µg/L HPβCD treatment was statistically different from the controls at a 95% significance interval, a 90 % significance level was chosen to reduce the chance of Type II statistical error, reducing the risk of missing an effect. The remaining HPβCD concentrations were not statistically significant different than the controls individually, although there still appeared to be an increase in GSI amongst all of the HPβCD treatments. Furthermore, it appeared that the increased GSI was an all-or-nothing response as most of the HPβCD treatments were statistically similar and did not respond in a concentration-dependent manner (Figure 18). As a result, all of the HPβCD treatments were pooled and compared to the controls (Figure 19). It was found that female flagfish exposed to HPβCD had a larger GSI than those not exposed ($P \leq 0.05$). This was a significant finding as it demonstrated that HPβCD may be stimulating the

gonad size in female flagfish. This effect was not detected in the GSI levels of male flagfish.

It has been noted that GSI may not be the most reliable endpoint in multi-spawning fish such as the American flagfish (Rinchard and Kestemont, 1996). A multi-spawning fish is defined as a fish that spawns on more than one occasion during a breeding period (Rinchard and Kestemont, 1996). Decreased reliability in GSI for multi-spawning fish is largely due to the variations in when the most recent spawning occurred relative to sampling taking place (Rinchard and Kestemont, 1996; Brewer *et al.*, 2008). A female that just laid eggs prior to sampling would be expected to have a lower GSI than a female that has not recently expelled eggs (Rinchard and Kestemont, 1996; Brewer *et al.*, 2008). This influence of mature egg weight on overall gonad weight can be a significant factor in calculating GSI. This is not often a concern in single spawning fish such as the roach (*Rutilus rutilus*), as a single spawning event can be controlled for (Rinchard and Kestemont, 1996). Although the variability in GSI for multi-spawning can be high, sampling a whole breeding harem often acts to reduce this variability as four females were sampled from each aquarium. By sampling multiple females from each aquarium and pooling replicates, there is a reduced chance that all females will have recently laid eggs, thus improving the accuracy of GSI. Sampling multiple females in addition to the consistent response observed amongst nearly all HPβCD treatments supports the hypothesis that HPβCD stimulated gonad size in female flagfish.

A potential mechanism for the apparent increase of female gonad size could be related to the ability for HPβCD to form inclusion complexes with steroid hormones, specifically progesterone. Progesterone is a vital hormone in the regulation of sperm and

oocytes maturation in fish (Kime, 1995). It has been demonstrated that a 2:1 HPβCD:progesterone inclusion complex is possible, improving the water solubility of progesterone (Zoppetti *et al.*,2007). The use of a HPβCD:progesterone complex has been shown to improve progesterone bioavailability in mammalian models, requiring a reduced dose of progesterone when used therapeutically (Zoppetti *et al.*, 2007). Due to the passive creation of HPβCD complexes, a HPβCD:progesterone complex may be occurring within the fish in the treated aquaria (Szejtli, 1998). Increases in the bioavailability of progesterone thus could potentially cause increased gonad size in female flagfish (Kime, 1995). Further research is needed to understand the impacts of HPβCD on the bioavailability of steroid hormones within fish.

5.2.4 Chronic Effects of HPβCD on Reproductive Output

The ability to reproduce is vital for the survival and maintenance of fish populations within an ecosystem. Monitoring reproductive output during chronic exposure to a toxicant can provide an abundance of information regarding the overall impacts a contaminant of interest is having on reproduction.

The time to first egg laying is an important factor to examine with regards to reproductive output (Sprague, 1973). It provides an insight into any effects a toxicant may be having on the ability of fish to reach sexual maturity (Sprague, 1973). Delays in reaching sexual maturity may cause fish that reproduce based on specific environmental cues to miss optimal spawning events (Rosenthal and Alderdice, 1976; Kime, 1995; Kime, 1999). These cues may include seasonal changes, alterations in water levels, and temperature fluctuations (Kime, 1995; Kime, 1999). When exposed to HPβCD, American flagfish showed no changes in time to first egg when compared to the controls (Table 4).

This result may have been influenced by the time chosen to thin each aquarium into breeding harems or when the breeding substrate was added. Setting up the breeding harems too late into their lifecycle may have caused any delays in reaching sexual maturity to be missed in this study.

Similarly, time to steady state spawning is a crude measure of the time required to reach sexual maturity. Time to steady state spawning is defined as the time required for an American flagfish to produce greater than 30 eggs in four consecutive days (Holdway and Dixon, 1986). This endpoint represents the length of time required for a flagfish to achieve consistently high egg output (Holdway and Dixon, 1986). Unlike time to first egg, time to steady state spawning is less vulnerable to missing delays in reaching sexual maturity based on experimental error. Time to steady state spawning generally occurs much later than time to first egg and is a representative of an established sexually mature breeding harem (Holdway and Dixon, 1986).

Fish in the 1600 µg/L HPβCD treatment reached steady state spawning faster relative to the controls (Table 4). This result was not consistent with the other HPβCD treatments (Table 4). This suggests effects on time to steady state spawning could be due to other factors such as differing behavioural interactions within each aquarium. The establishment of dominance between the two males in each tank can vary. Due to the fact that dominance is largely based on size and aggression, males that are more evenly matched may require longer time to establish dominance (St Mary *et al.*, 2001). Delays in dominance can influence the time to steady state spawning and may be misinterpreted as a delay in sexual maturity.

The total number of days during the 30 day breeding period during which eggs were laid was variable between the controls and the HPβCD treatments (Table 4). With the exception of the 5, 50, and 500 μg/L HPβCD treatments, breeding occurred on more days in harems exposed to HPβCD. This finding should be interpreted with caution for similar reasons as the time to first egg laying results, as establishing dominance may have played a role in these discrepancies.

The total number of eggs produced is a measure of the total reproductive output over the course of the breeding period. This endpoint provides information regarding the reproductive capacity of the flagfish (Sprague, 1973). There were no significant differences in the total number of eggs produced relative to controls, although interestingly 1600 μg/L HPβCD exposed fish produced the greatest number of eggs during the breeding period (Table 4). No observed overall differences in total egg output along with the observed high egg output in the 1600 μg/L HPβCD treatment represents an intriguing finding as it demonstrates that even high exposure concentrations of HPβCD did not inhibit egg production in the American flagfish.

Mean daily egg production, representing the average egg production for only days in which eggs were laid was also examined (Holdway and Dixon, 1986). This analysis was used to account for the difference in total egg production due to the variance in the total number of days in which eggs were laid. By accounting for factors such as delayed time to first egg laying, daily egg production can be analyzed. Similar to total egg production, there was no significant difference in mean daily egg production between any of the HPβCD treatments and the controls (Table 4). The fish in treatments containing

1600 µg/L HPβCD experienced the highest mean daily egg production, again suggesting that HPβCD does not impede reproductive output of American flagfish.

5.2.5 Chronic Effects of HPβCD on Reproductive Viability

Reproductive viability was monitored during the breeding period. Percent fertilized, percent hatch, time to hatch, and incidents of malformations were monitored for all eggs produced.

Percent fertilization is important in assessing the reproductive success of fish. Alterations in the percent of fertilized eggs provide indications of reproductive impairment originating from either the quality of male or female gametes (Lahnsteiner and Leitner, 2013). Given that American flagfish are oviparous, fertilization of eggs occurs outside of the body, and alterations in physical breeding behaviour may also affect the percent fertilization (Fogels and Sprague, 1977). Following chronic exposure to various concentrations of HPβCD, a reduction in percent fertilization was observed in the 5 µg/L of HPβCD treatment (Table 5). This effect was not observed at any of the other HPβCD concentrations tested. This reduction in fertilization only at a low concentration of HPβCD may be due to several factors. One of which could be the result of a HPβCD concentration that is too low to initiate detoxification pathways within the parental flagfish, also known as low dose inhibition of fertilization (Calabrese and Baldwin, 2001).

Percent hatch and time to hatch both represent general egg quality and viability. A reduced time to hatch may result in a decreased likelihood of offspring survival (Sprague, 1971; Rosenthal and Alderdice, 1976). On the other hand, failure to hatch will directly

impact the survival of offspring. Chronic exposure of American flagfish to HPβCD over a full life-cycle did not influence either the time to hatch or the percent hatch of their offspring.

Increased incidence of offspring malformations may impact the long-term survival of offspring within the wild (Nesan and Vijayan, 2012). A tenfold increase in the incidence of offspring malformations was observed in the 160 µg/L HPβCD treatment, relative to controls (Figure 21). This was not observed in higher or lower concentrations of HPβCD. The observed malformations that occurred at 160 µg/L of HPβCD were similar in appearance to the malformations that occurred, less frequently, in the other treatments and controls. They were characterized as a bent spine and appeared to be pericardial edema due to the collection of blood near the heart (Wang *et al.*, 2013). It has been shown that stress can induce a rapid increase in circulating cortisol in fish, and increases in cortisol levels in parental zebrafish have been linked to an increase in the incidence of pericardial edema in their offspring (Castranova *et al.*, 2005; Nesan and Vijayan, 2012). Thus the observed malformations observed in the offspring may be due to the presences of a non-chemical stressor. Stressors that the breeding harems may have encountered over the course of the experiment include: daily vacuuming, daily removal of breeding substrate as well as establishment of dominance between males within a confined space. The daily aquarium maintenance may explain the background occurrence of malformation within the controls and HPβCD treatments (Figure 21). However, a significant dispute of dominance between the males occurred in one of the 160 µg/L of HPβCD aquaria at approximately the same time as the increased incidence of malformations. This dispute lasted over an hour and left the male exhausted and the

females huddled in the rear of the aquarium. It is unknown if a similar dispute occurred within any other aquaria. However, the stress induced from this fight may have increased the cortisol levels of the fish within this aquarium, thereby resulting in an increase in the presence of malformations. Based on these observations, it is likely that the increased occurrence of malformations at 160 µg/L of HPβCD was due to behavioural stress rather than HPβCD exposure.

5.2.6 Chronic Effects of HPβCD Exposure on Offspring Growth and Survival

Offspring from parental flagfish chronically exposed to HPβCD over one full life-cycle were reared for 30 DPH in the same manner as their parents. While experiencing the same HPβCD treatments as their parents, the offspring experienced no discernible difference in mortality when compared to the controls (Table 6). This indicates that mortality was not affected when exposed to HPβCD for two generations.

Length was monitored at 21 and 28 DPH during the 30 day second generation rearing period. At 21 DPH all HPβCD treatments other than 1600 µg/L were smaller than the controls (Figure 22). At 28 DPH, the controls had the greatest total length; however they were only statistically different from the 500 µg/L HPβCD treatments (Figure 23). This suggests that any difference in length observed in the second generation at 21 DPH had reduced by 28 DPH, indicating in the effects of HPβCD on total length may be temporary. Reduction in growth may alter the stage of development or level of maturation, potentially leading to an increase to the susceptibility to predation and disease (Sprague, 1971). Also, alterations in growth may influence overall fitness of organisms within an ecosystem (Rosenthal and Alderdice, 1976). It is important to note that growth may be influenced by many factors such as fish density, temperature, food availability, or

toxicant exposure (Sprague, 1971). Although growth analysis is important, it is more useful as a pre-screening tool rather than as a sensitive endpoint of toxicity (Woltering, 1984).

5.2.7 Chronic Effects of HPβCD Exposure on Offspring Sensitivity to Copper

Offspring of fish treated with HPβCD were challenged with exposure to the reference toxicant copper, to assess two important characteristics of HPβCD. Firstly, it was used to determine if the parental exposure to HPβCD influenced the tolerance to another toxicant (Adeyemi and Klerks, 2013). Secondly, alterations in sensitivity may provide insight into whether or not HPβCD is metabolically active based on upregulation of detoxification enzymes (Kashian, 2004). It was found that larval flagfish from parents exposed to HPβCD experienced a general reduction survival when exposed to copper (Figure 24). This decreased tolerance, however, was not observed at high copper concentrations in offspring of parents exposed to 1600 μg/L HPβCD.

The results suggest that parental exposure to HPβCD may increase offspring sensitivity to other toxicants. Typically, when a toxicant is metabolically active, an organism becomes less sensitive to a reference toxicant (Kashian, 2004). However, this was not the case in this study, as the results indicate that HPβCD may be metabolically active in a different fashion to copper in the American flagfish. One possibility is that an increase in the metabolic costs used in HPβCD detoxification may take away from the energy reserves required for the metabolic detoxification of copper.

It is well understood that copper induces ionoregulatory impairment in fish, inhibiting Na^+/K^+ ATPase in the gill (Adeyemi and Klerks, 2013). This often results in

damage of the sodium pump and therefore a reduced ability to regulate sodium levels within the organism; this can lead to osmatic stress and often results in death at low concentrations (Adeyemi and Klerks, 2013). It is thought that copper is detoxified through the induction of specific low molecular weight proteins known as metallothioneins (Adeyemi and Klerks, 2013). These proteins are used in the detoxification of non-essential metals, while regulating essential metals (Adeyemi and Klerks, 2013). It has been shown that pre-exposure to copper results in acclimation and reduced toxicity in killifish, likely due to induction of metallothionein production (Adeyemi and Klerks, 2013).

Metallothionein detoxification appears to be specific to metals and requires a high energy demand (Adeyemi and Klerks, 2013; Lindeque *et al.,* 2013). Mice with a metallothionein knockout become obese and have higher anabolic energy stores, indicating that metallothionein is an energy intensive process (Lindeque *et al.,* 2013). Given that HPβCD is not a metal, the results are consistent with the finding from this current study, and HPβCD more than likely utilizes a different detoxification pathway than copper. This indicates that induction of metallothionein detoxification in offspring from HPβCD exposed fish subsequently exposed to low concentrations of copper may be impaired or inhibited. The reduction in copper detoxification may be due to the energy investment put into HPβCD detoxification rather into high energy demand metallothionein production (Lindeque *et al.,* 2013). This again suggests that HPβCD may be metabolically active within the American flagfish, however the pathway is currently unknown.

5.3 Current Limitations and Future Directions

Although this study was the first of its kind to examine the effects of HPβCD on an aquatic species, there were some limiting factors which may have altered the results. Because of this, conducting a complete second generational reproductive study would have been valuable in understanding the effects of HPβCD on flagfish. Identifying the influence of HPβCD on second generation GSI would have helped confirm the current findings, if time had permitted. Additionally, examining the histo-pathological and biochemical changes in female gonads would have been of value. Identifying any changes that HPβCD may have on the yolk precursor protein vitellogenin would help confirm the observations found in this study. Also, explicit rationing of food could have been important in confirming that HPβCD has no effect on growth. Examining vitellogenin levels in females exposed to HPβCD would help confirm the increases in female gonad size observed in this study.

Furthermore, HPβCD is a prime candidate for combinatorial toxicity studies due to the presence of a non-specific binding cavity. The ability to improve the solubility and bioavailability of countless pharmaceuticals and toxicants makes it a very intriguing compound to toxicologists. As shown, there are many more avenues which need to be examined with regards to the potential effects that HPβCD has on American flagfish and other aquatic species.

6.0 Conclusion

Understanding the effects of pollutants on non-target biota has been a goal for environmental toxicologists for many years. As of late, the impact of pharmaceuticals and personal care products (PPCPs) has become a very intriguing topic. Being inherently biologically active, they have driven an increase in the study of the non-target effects of PPCPs within aquatic ecosystems. Of interest in this study were the effects of hydroxylpropyl-β-cyclodextrin (HPβCD) on American flagfish (*Jordanella floridae*). In this study, a short-term exposure, and a complete life-cycle study were completed in order to uncover any non-target effects that HPβCD may be having on fish.

It was found that HPβCD has no effect on survival of the larval American flagfish when acutely exposed to concentrations of up to 1 g/L of HPβCD. Additionally, when chronically exposed to lower, environmentally relevant concentrations of HPβCD, no mortality was observed. Importantly, it was discovered that HPβCD resulted in an increase in the gonad size of sexually mature female flagfish in an all or nothing response, regardless of HPβCD concentration. Furthermore, it was found that offspring from parents exposed to HPβCD were moderately more sensitive when challenged with copper.

Although there was no significant impact on the survival, growth, and total reproductive output, stimulation in female gonad size occurred. This result, combined with an increase in sensitivity to copper as a reference toxicant suggests that HPβCD may not be toxicologically benign to non-target organisms even at low concentrations, providing many avenues for future research.

Appendix 1: Nominal and actual concentrations of HPβCD from the full life-cycle study.

Replicate	Nominal HPβCD Concentrations (µg/L)	Actual HPβCD Concentrations (µg/L)
A	0	--
A	5	--
A	16	--
A	50	--
A	160	--
A	500	--
A	1600	--
B	0	--
B	5	--
B	16	--
B	50	--
B	160	--
B	500	--
B	1600	--

-- Results Pending

Appendix 2: Time line of activity and observations during the life-cycle exposure of HPβCD to American flagfish

Time	**Activity**	**Observation**
N/A	Collection of eggs to be used in chronic life-cycle exposure	
Egg	Begin Exposure to HPβCD	Monitor hatching and survival
10 DPH	Transfer to 1 L polypropylene Container	Monitor survival
21 DPH	Growth Measurement	Photo Measured Length
28 DPH	Growth Measurement	Photo Measured Length
30 DPH	Transfer to 70 L Aquarium	Monitor Survival
77- 74 DPH	Thin to 15 fish per treatment	Length, weight, condition factor, HSI
102-103 DPH	Thin to breeding harem	Length, weight, condition factor, HSI, GSI
109-139 DPH	Egg collection	Egg Production, % Fertilization, % hatch, % malformed
136-139 DPH	Copper Challenge	Monitor survival
144-145 DPH	Final Dissection	Length, weight, condition factor, HSI, GSI
138-172 DPH	Second Generation Rearing	Monitor hatching and Survival

Note: Time (DPH) is in reference to parental flagfish exposed to HPβCD

References

Adeyemi, J. and Klerks, P. (2013). Occurrence of copper acclimation in the least killifish *Heterandria Formosa*, and associated biochemical and physiological mechanisms. *Aquatic Toxicology, 130-131: 51-57.*

Batt, A., Kim. S., and Aga, D. (2007). Comparison of the occurrence of antibiotics in four full-scale wastewater treatment plants with varying designs and operations. *Chemosphere, 68: 428-435.*

Beyger, L., Orrego, R., Guchardi, J., and Holdway, D. (2012). The acute and chronic effects of endosulphan pulse-exposure on *Jordanella floridae* (Florida flagfish) over one complete life-cycle. *Ecotoxicology and Environmental Safety, 76:71-78.*

Brewer, S., Rabeni, C., and Papoulias, D. (2007). Comparing histology and gonadosomatic index for determining spawning condition of small-bodied riverine fishes. *Ecology of Freshwater Fish, 17: 54-58.*

Calabrese, E. and Baldwin, L. (2001). Hormesis: U-shaped dose responses and their centrality in toxicology. *Trends in Pharmacological Sciences, 22: 285-295.*

Carballa, M., Omil, F., Lema, J., Llompart, M., Garcia-Jares, C., Rodriguez, I., Gomez, M., and Terns, T. (2004). Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. *Water Research, 38: 2918-2926.*

Castranova, D., King, W., and Woods, C. (2005). The effects of stress on androgen production, spermination response and sperm quality in high and low cortisol responsive domesticated male striped bass. *Aquaculture, 246: 413- 422.*

Caux, P., Kent, R., Fan G., and Stephenson, G. (1996). Environmental fate and effects of chlorothalonil: A Canadian Perspective. *Chritical Reviews in Environmental Science and Technology, 26: 45-93.*

Cleuvers, M. (2004). Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. *Ecotoxicology and environmental safety, 59: 309-315.*

Corcoran, J., Winter, M., and Tyler, C. (2010). Pharmaceuticals in the aquatic environment: A critical review of the evidence for health effects in fish. *Critical Reviews in Toxicology, 40(4): 287-304.*

Daughton, C. and Ternes, T. (1999). Pharmaceuticals and personal care products in the environment: agents of subtle change? *Environmental Health Perspectives, 107: 907-938.*

Davis, M. and Brewster, M. (2004). Cyclodextrin-based pharmaceutics: past present and future. *Nature Reviews, 3: 1023-1035.*

Del Valle, M. (2004). Cyclodextrins and their uses: a review. *Process Biochemistry, 39: 1033-1046.*

Durán-Alvarez, J., Becerril-Bravo, E., Silva Castro, V., Jiménez, B., and Gibson R. (2009). The analysis of a group of acidic pharmaceuticals, carbamazepine, and potential endocrine disrupting compounds in wastewater irrigated soils by gas chromatography–mass spectrometry. *Talanta, 78: 1159-1166.*

Fogels, A. and Sprague, J. (1977). Comparative short-term tolerance of zebrafish, flagfish, and rainbow trout to five poisons including potential reference toxicants. *Water Research, 11: 811-817.*

Foster, N., Cairns, J., and Kaesler, R. (1969). The flagfish, *Jordanella floridae*, as a laboratory animal for behavioural bioassay studies. *Proceedings of the National Academy of Science Philadelphia, 121: 129-152.*

Gould, S. and Scott, R. (2005). 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD): A toxicology Review. *Food and Chemical toxicology, 43: 1451-1459.*

Hedges, A. (1998). Industrial applications of cyclodextrins. *Chemical Reviews, 98: 2035-2044.*

Holdway, D., Sprague, J., and Dick, J. (1982). Bioconcentration of vanadium in American flagfish over one reproductive cycle. *Water Research, 17: 937-941.*

Holdway, D. and Dixon, D. (1986). Impact of pulse exposure to methoxychlor on fagfish (*Jordanella floridae*) over one reproductive cycle. *Canadian Journal of Fisheries and Aquatic Sciences, 43: 1410-1415.*

Hoon, T., Dawood, Y., Kahn-Dawood F., Ramos, J., and Batenhorst, R. (1993). Bioequivalence of 17β-estradiol hydroxypropyl-β-cyclodextrin complex in postmenopausal women. *The Journal of Clinical Pharmacology, 33: 1116-1121.*

Hui, W., Xiaoxia, X., Fengyi, Z., Qian, Z., Qing, T., Yina, Z., Cheng, C., Chengliang, Z. and Shuqin, Y. (2011). Evaluation of cholesterol depletion as a marker of nephrotoxicity *in vitro* for novel β-cyclodextrin derivatives. *Food and Chemical Toxicology, 49: 1387-1393.*

IMS Health (2012). Total Unaudited and Auted Global Pharmaceutical Markey, 2003 – 2011. Retreived May 27, 2013. <http://www.imshealth.com/deployedfiles/ims/Global/Content/Corporate/Press%20Room/Top-Line%20Market%20Data%20&%20Trends/2011%20Top-line%20Market%20Data/Global_Pharma_Market_by_Spending_2003-2011.pdf>

Kanda, R., Griffin, P., James, H., and Fothergill, J. (2003). Pharmaceuticals and personal care products in sewage treatment. *Journal of Environmental Monitoring, 5: 823-830.*

Kashian, D. (2004). Toxaphene detoxification and acclimation in *Daphnia magna*: do cytochrome P-450 enzymes play a role? *Comparative Biochemistry and Physiology Part C, 137: 53-63.*

Kaushik, S. (1998). Nutritional bioenergetics and estimation of waste production in non-salmonids. *Aquatic Living Resources, 11: 211-217.*

Kidd, K., Blanchfield, P., Mills, K., Palace,V., Evans, R., Lazorchak, J., and Flick, R. (2007). Collapse of a fish population after exposure to a synthetic estrogen. *Proceedings of the National Academy of Sciences, 104: 8897-8901.*

Kime, D. (1995). The effects of pollution on reproduction in fish. *Reviews in Fish Biology and Fisheries, 5: 52-96.*

Kime, D. (1999). A strategy for assessing the effects of xenobiotics on fish reproduction. *The Science of the Total Environment, 225: 3-11.*

Kitamura, S., Shimizu, Y., Yoshida, Y., Sugihara, K., and Ohta, S. (2001). Reductive metabolism of *p,p'*-DDT and *o,p'*-DDT by rat liver cytochrome P450. *The American Society of Pharmacology and Experimental Therapeutics, 30: 113-118.*

Klug, H., Chin, A., and St Mary, C. (2003). The net effects of guarding on egg survivorship in the flagfish, *Jordanella floridae. Animal Behaviour, 69: 661-668.*

Lahnsteiner, F. and Leitner, S. (2013). Effects of temperature of Gametogenesis and Gamete Quality in brown trout, *Salmo trutta. Journal of Experimental Zoology, 319: 138-148.*

Leeson, P. and Springthorpe, B. (2007). The influence of drug-like concepts on decision-making in medicinal chemistry. *Nature Reviews, 6: 881-890.*

Lewis, M. (1992). The effects of mixtures and other environmental modifying factors on the toxicities od surfactants to freshwater and marine life. *Water Research, 26: 1013-1023.*

Lindeque, J., Hidalgo, J., Louw, R. and Westhuizen, F. (2013). Systemic and organ specific metabolic variation in metallothionein knockout mice challenged with swimming exercise. *Metabolomics, 9: 418-432.*

Loftsson, T. and Brewster, M. (2011). Pharmaceutical application of cyclodextrins: effects on drug permeation through biological membranes. *Pharmacy and Pharmacology, 63: 1119-1135.*

Marsalek, J. (2008). Pharmaceuticals and personal care products (PPCP) in Canadian urban waters: a management perspective. *Environment Canada, National Water Research Institute, 867 Lakeshore Road, Burlington ON. L7R 4A6, Canada.*

Meylan, W., Howard P., Boethling, R., Aronson, D., Printup, H. and Gouchie, S. (1998). Improved method for estimating bioconcentration/bioaccumulation factor from octanol/water partition coefficient. *Environmental Toxicology and Chemistry, 18: 664-672.*

Monteiro, S. and Boxall, B. (2010). Occurance and fate of human pharmaceuticals in the environment. *Reviews of Environmental Contamination and Toxicology, 202: 53-154.*

Nesan,D. and Vijayan, M. (2012). Embryo exposure to elevated cortisol level leads to cardiac performance dysfunction in zebrafish. *Molecular and Cellular Endocrinology, 363: 85-91*.

Nichols, J., Erhardt, S., Dyer, S., James, M., Moore, M., Plotzke, K., Segner, H., Schultz, I., Thomas, K., Vasiluk, L. and Weisbrod, A. (2007). Use of in vitro absorption, distribution, metabolism, and excretion (ADME) data in bioaccumulation assessments for fish. *Human and Ecological Risk Assessment, 13: 1164-1191*.

Pasquini, L., Munoz, J., Rimlinger, N., Dauchy, X., France, X., Pons M. and Gorner, T. (2013). Assessment of the fate of some household micropollutants in urban wastewater treatment plant. *Chemical Papers, 67: 601-612.*

Ratola, N., Cincinelli, A., Alves, A., and Katsoyiannis, A. (2012). Occurrence of organic microcontaminants in the wastewater treatment process. A mini review. *Journal of Hazardous Materials, 239-240: 1-18.*

Rasheed, A., Kumar, A. and Sravanthi, V. (2008). Cyclodextrins as a drug carrier molecule: A review. *Scientia Pharmaceutica, 76: 567-598.*

Rinchard, J. and Kestemont, P. (1996). Comparative study of reproductive biology in single- and multiple-spawner cyprinid fish. I. Morphological and histological features. *Journal of Fish Biology, 49: 883-894.*

Rosenthal, H. and Alderdice, D. (1976). Sublethal effects of environmental stressors, natural and pollutional, on marine fish eggs and larvae. *Journal of Fisheries Research Board of Canada, 33: 2047-2065.*

Rosi-Marshall, E. and Royer, T. (2012). Pharmaceutical compounds and ecosystem function: an emerging research challenge for aquatic ecologists. *Ecosystems, 15: 867-880.*

Sadekarpawar, S., and Parikh, P. (2013). Gonadosomatic and hepatosomatic indices of freshwater fish *oreochromis mossambicus* in response to a plant nutrient. *World Journal of Zoology, 8: 110-118.*

Sherwood, G., Rasmussen, J., Rowan, D., Brodeur, J., and Hontela, A. (2000). Bioenergetic costs of heavy metal exposure in yellow perch (*Perca flavescens*): in situ estimates with radiotracer (^{137}Cs) technique. *Canadian Journal of Fisheries and Aquatic Sciences, 57: 441-450.*

Simonich, S. and Hites R. (1995). Organic Pollutant Accumulation in Vegetation. *Environmental Science and Technology, 28: 2905-2914.*

Smolders, R., Bervoets, L., De Boeck, G., and Blust, R. (2002). Integrated condition indices as a measure of whole effluent toxicity in zebrafish (*Danio rerio*). *Environmental Toxicology and Chemistry, 21: 87-93.*

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

Sprague, J. (1969). Measurement of pollutant toxicity to fish I.* Bioassay methods for acute toxicity. *Water Research Pergamon Press, 3: 793-821.*

Sprague, J. (1971). Measurement of pollutant toxicity to fish. III. Sublethal effects. *Water Research, 5: 245-266.*

Sprague, J. (1973). The ABC's of pollutant bioassay using fish. *Biological Methods for the Assessment of Water Quality, ASTM STP 528,* American Society for Testing and Materials, 6-30.

St Mary, C., Noureddine, C., and Lindstrom, K. (2001). Environmental effects on male reproductive success and parental care in the Florida flagfish (*Jordanella floridae*). *Ethology, 107: 1035-1052.*

Szejtli, J. (1998). Introduction and general overview of cyclodextrin chemistry. *Chemical Reviews, 98: 1743-1753.*

Szente, L. and Szejtli, J. (1999). Highly Soluble cyclodextrin derivatives: chemistry, properties, and trends in development. *Advanced Drug Delivery Reviews, 36: 17-28.*

Taylor, G., Weiss, J., and Pitha, J. (1989). Testosterone in cyclodextrin-containing formulation: behavioral and physiological effects of episode-like pulses in rats. *Pharmaceutical Research, 6: 641-646.*

van der Oost, R., Beyer, J., and Vermeulen, N. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology, 13: 57-149.*

Wang, K., Ma, J., He, M., Gao, G., Xu, H., Sang, J., Wang, Y., Zhao, B., and Cui, D. (2013). Toxicity Assessments of near-infrared upconversion luminescent LaF_3:Yb,Er in early development of Zebrafish embryos. *Theranostics, 3: 258-266.*

Whyte, J., Jung, R., Schmitt, C., and Tillitt, D. (2000). Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure. *Critical Reviews in Toxicology, 30: 347-570.*

Woltering, D. (1984). The growth response in fish chronic and early life stage toxicity tests: A critical review. *Aquatic Toxicology, 5: 1-21.*

Yang, J. and Metcalfe, C. (2006). Fate of synthetic musks in a domestic wastewater treatment plant and in an agricultural field amended with biosolids. *Science of the Total Environment, 363: 149-165.*

Yuan, C. and Jin, Z. (2007). Aerobic biodegradability of hydroxypropyl-β-cyclodextrin in soil. *Journal of Inclusion Phenomena and Macrocyclic Chemistry, 58: 345-351.*

Zoppetti, G., Puppini, N., Pizzutti, M., Fini, A., Giovani, T., and Comini, S. (2007). Water soluble progesterone-hydroxypropyl-β-cyclodextrin complex for injectable formulations. *Journal of Inclusion Phenomena and Macrocyclic Chemistry, 57: 283-288.*