AN ABSTRACT OF THE THESIS OF Andres Joshua Reyes for the Master of Science in Biology presented May 12, 2017.

Title: *SIGANUS SPINUS* (SCRIBBLED RABBITFISH) AS A SENTINEL SPECIES FOR ASSESSING XENOBIOTIC POLLUTION IN TROPICAL MARINE ECOSYSTEMS

Approved: _____

Jason S. Biggs, Chairman, Thesis Committee

Pharmaceuticals and personal care products (PPCPs) play a role in mitigating disease and improving the quality of human life. However, PPCPs are potential environmental pollutants of concern because they (i) can induce physiological effects, (ii) are long-lived in the environment, and (iii) are prevalent in nearly all sewage treatment plant effluent. Managing PPCP pollution is therefore one critical aspect of natural resource management that needs to be addressed. Specifically, a need arises to determine how foreign compounds (i.e., xenobiotics) are changing the biological activity of organisms within marine ecosystems. This thesis examined the *Siganus spinus*, scribbled rabbitfish, hepatic cytochromeP450 (CYP) system to quantify changes in CYP-mediated catabolic activity to demonstrate potential consequences of pharmacopollution in tropical coral reefs. *S. spinus* hepatic CYP1A-mediated activity increased significantly, following a single, 24-hour exposure to two xenobiotics - β-naphthoflavone (BNF) and 17α-ethinylestradiol (EE2) – intraperitoneal and within the surrounding seawater, quantified using the 7-ethoxyresorufin-O-deethylase assay. Interestingly, however, hepatic CYP3A-

mediated activity also increased significantly, but only in the groups exposed to EE2 intraperitoneal and within the surrounding seawater, quantified using the 7-methoxyresorufin-O-deethylase assay. Total protein concentrations were quantified to assess the potential metabolic consequences of *S. spinus*. Exposure to the xenobiotics significantly decreased total protein concentrations, suggesting diminished hepatic physiology following exposure, quantified using the Bradford assay. Although significant attenuated total protein concentrations were observed in the groups exposed to the xenobiotics, BNF and EE2 significantly increased hepatic CYP1A-mediated activity, whereas only EE2 significantly increased CYP3A-mediated activity. Our findings demonstrate that *S. spinus* CYPs respond to xenobiotics which can serve as ecologically-relevant biomarkers to quantify biological change(s) in tropical marine ecosystems throughout Micronesia.

TO THE OFFICE OF GRADUATE STUDIES

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SIGANUS SPINUS (SCRIBBLED RABBITFISH) AS A SENTINEL SPECIES

FOR ASSESSING XENOBIOTIC POLLUTION

IN TROPICAL MARINE ECOSYSTEMS

BY

ANDRES JOSHUA REYES

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<u>Acknowledgments:</u>

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Introduction

Chemical Ecology in Marine Environments:

Diverse chemical compounds play critical roles in the ecology of many organisms, and can influence the community structure of entire ecosystems (Agrawal, 2004; Paul et al., 2007; Paul and Ritson-Williams, 2008; Sotka et al., 2009; Paerl, 1988; Noga et al., 1996; Nagle and Paul, 1998). This complexity reaches a peak on tropical coral reefs, which are well-known targets of bioprospecting agents. Although many marine natural products have been studied for their potential biomedical applications, and much is known about their mechanisms of action in humans, much less is understood regarding their roles in ecology.

In many ways, organisms have evolved competitive, offensive, and/or defensive strategies to live in dense coral reef habitats. One such strategy prevalent throughout marine realm is the production of organic compounds that serve no physiological roles within their makers, otherwise known as 'secondary metabolites' (Hay and Fenical, 1988; Paul and Van Alstyne, 1992; Pawlik, 1993; Berry et al., 2008). Secondary metabolites produced within marine ecosystems fall into a number of chemical classes (e.g., terpenes, acetogenins, alkaloids, halogenated hydrocarbons, and polyphenols), but the vast majority discovered to date are small hydrophobic molecules that have a tendency to induce biological activity or accumulate in other living things. These chemicals, which are foreign to the body, are generally referred to as xenobiotics. And because secondary metabolites are compounds that are not directly involved in the normal growth, development or reproduction of an organism, they can be considered as good examples of

xenobiotics because their role is to exert biological activity upon other organisms in ways that often gain ecological advantages for their users.

Pollutants have the Potential to Influence Ecology:

Manmade chemicals that enter the environment are also good examples of xenobiotics because they also can alter biological activity within organisms. The response of consumers to xenobiotics can ultimately influence their health, reproduction, and survival. Yet, for tropical marine ecosystems, almost nothing is known regarding organisms' abilities to overcome the increasing diversity of bioactive compounds encountered within coastal habitats. Is it possible that a drug-drug interaction exists among marine natural products and manmade organopollutants? A strong argument for this possibility will unfold throughout this thesis.

Even in the absence of anthropogenic influence, many marine consumers are continually being exposed to naturally-occurring chemicals found within their prey. Understanding how predators cope with prey chemical defenses is not only needed to learn how biochemistry structures ecosystems (molecular ecology), but also to guide efforts to create effective drugs for diseases that have no cure (i.e., drug discovery and design). Additionally, knowledge of the mechanisms conferring consumer tolerance to diet-derived chemicals is relatively sparse for marine systems (Vrolijk and Targett, 1992; Sotka and Whalen, 2008, Sotka et al., 2009) and there is much more to be discovered.

Although it is extremely difficult to determine the mechanism of action of a novel compound, one way that a meaningful biological response can be measured - in organisms including humans - is through enzymes that have documented, similar roles in the detoxification and clearance of foreign substances; i.e., "xenobiotic metabolizing

enzymes" (XMEs). Some XMEs have proven useful as 'biomarkers' for monitoring terrestrial and freshwater ecosystems because their substrates rarely occur naturally. One such widely employed biomarker is the cytochrome P450 system for which numerous assays have been developed for monitoring exposure to polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides, personal care products, pharmaceuticals, and other similarly-classed, persistent organic pollutants (Nilsen et al., 1998; Boxall, 2004). In fact, many enzymes within the cytochrome P450 (CYP or CYP450) superfamily have an extensive array of substrates, isoforms, and assays to measure changes in their gene expression, protein concentrations, and/or catalytic activities.   Collectively, such techniques serve as informative tools for demonstrating and monitoring the ecological consequences of being exposed to broad classes of foreign compounds called xenobiotics.

Sources of Pharmaceutical Pollution:

Because they are designed to influence physiology (e.g., disease states) in humans, pharmaceuticals and personal care products (PPCPs) are classified as potential environmental pollutants of concern. Conservation of functional genes and biochemical pathways throughout evolution suggests that biological activity is also possible in a wide range of wild organisms; including those in marine environments (Boxall, 2004; Fent et al., 2006; Corcoran et al., 2010; Daughton and Ternes, 1999). The potential for this is underscored by the discovery that PPCPs are prevalent in nearly all sewage treatment plant (STP) effluent, suggesting that ecosystems adjacent to outfalls may already be affected (Andreozzi et al., 2003). For example, 17α-ethinylestradiol (EE2), a synthetic steroid commonly used in oral contraceptives, caused complete reproductive failure in

Canadian fathead minnows (*Pimephales promelas*) after long-term, chronic exposure to municipal effluent-laden waterways (Kidd et al., 2007). In Saipan (CNMI), estrogenic activity detected within Tanapag Lagoon (estrogen equivalence: 0.03–31.80ng/L) was linked to the presence of bisphenol A (10.5–182ng/L) and estrone (0.05–0.1ng/L) emanating from two nearby sewage outfalls (Harrison, 2008). By interacting with the enzymes responsible for their detoxification, xenobiotics released into the environment by STP effluents have the potential to change the fate of chemicals naturally encountered within the diets of marine herbivores. This is a drug-drug interaction that has yet to be addressed in wild ecosystems, terrestrial or marine. Understanding, documenting, and monitoring the effects of pharmacopollution is therefore one critical aspect of natural resource management that needs to be addressed in coastal marine ecosystems throughout Micronesia.

Cytochrome P450 Gene Expression:

CYP450 genes respond to xenobiotics, making them ecologically-relevant biomarkers. Although some compounds can knock out CYP activity directly, these enzymes are rarely the ultimate physiological target of a drug or xenobiotic. Instead, CYP-genes are typically induced by their exposure to xenobiotics and sustained CYP activity can eventually shift metabolic pathways. This study capitalizes on our ability to measure changes in CYP-isoform-mediated metabolic activity as a way to demonstrate potential consequences of pharmacopollution in tropical coral reef ecosystems.

Previous studies have demonstrated that a classical aryl-hydrocarbon receptor agonist, β-naphthoflavone (BNF), induced the hepatic CYP1A detoxifying pathway of the scribbled rabbitfish (*Siganus spinus*) in laboratory trials (Emborski et al., (2012). As

would be expected of a system sensitive to the presence of foreign compounds, CYP1A activity was both dose and time dependent. Following a single intraperitoneal injection of BNF, hepatic CYP1A activity and protein abundance reached their maximum less than two days after exposure and returned to baseline after sixteen (Emborski et al., 2012). These findings were the first to suggest that tropical marine herbivores had the ability to detect and detoxify xenobiotics; i.e., *S. spinus* maintained genetic conservation of the inducible aryl-hydrocarbon detoxifying pathway. The present thesis builds upon the findings of Emborski et al., (2012) by expanding the focus to both an environmentally relevant PPCP, 17α-ethinylestradiol (EE2), and an ecologically-relevant route of exposure - passive absorption from the surrounding seawater.

Null Hypotheses:

H_{o1}: The intraperitoneal exposure of the xenobiotic EE2 exhibits no significant effects on CYP-meadiated catalytic activity of *S. spinus* hepatic microsomes when compared to vehicle control groups. H_{o1} will be proven false if a one-way analysis of variance (ANOVA) and *post-hoc* analyses indicate significant differences between the vehicle control versus the in-water and intraperitoneal treatment groups.

H_{o2}: The in-water exposure of the xenobiotics EE2 or β-naphthoflavone (BNF) exhibits no significant effects on the metabolic activity of *S. spinus* hepatic microsomes compared to vehicle control groups. H_{o2} will be proven false if a one-way ANOVA and *post-hoc* analyses indicate significant differences between the vehicle control versus the in-water and intraperitoneal treatment groups.

H_{o3}: Intraperitoneal and/or in-water exposure of EE2 or BNF exhibit no significant effects on the total protein concentration of *S. spinus* hepatic microsomes

compared to vehicle control treatment groups. H_{o3} will be proven false if a one-way ANOVA and *post-hoc* analyses indicate significant differences between the vehicle control versus the in-water and intraperitoneal treatment groups.

Methods

S. spinus Collection:

S. spinus adults were collected from Rios Beach, Guam (13.4443° N, 144.7937° E; Figure 1) on October 24, 2015. Fish collections coincided with lunar cycles causing low-tides to occur between 20:00 and 23:00 h. Attention was given to obtaining adults of the same size (~12 cm fork length). Fish were caught live by hand using 12"x10" minnow nets and temporarily housed in live-wells specifically designed for the task: neutrally-buoyant 20-L containers with the lids and bottoms modified with mesh to allow water to exchange freely, while also protecting against wave surge. At the end of collections, fish were then brought to shore and transferred into a 200-L aerated tank for transport to the University of Guam Marine Laboratory (UOGML). Fish were placed into a 1400-L fiberglass tank immediately upon arrival at the UOGML, and supplied with a constant source of seawater within the UOGML flow-through system. Stress on these wild-caught fish was minimized by preventing them from ever being out of water and minimizing the time spent in static tanks. As collections on Guam often occur less than 40 minutes from the UOGML, no fish were lost under these conditions.
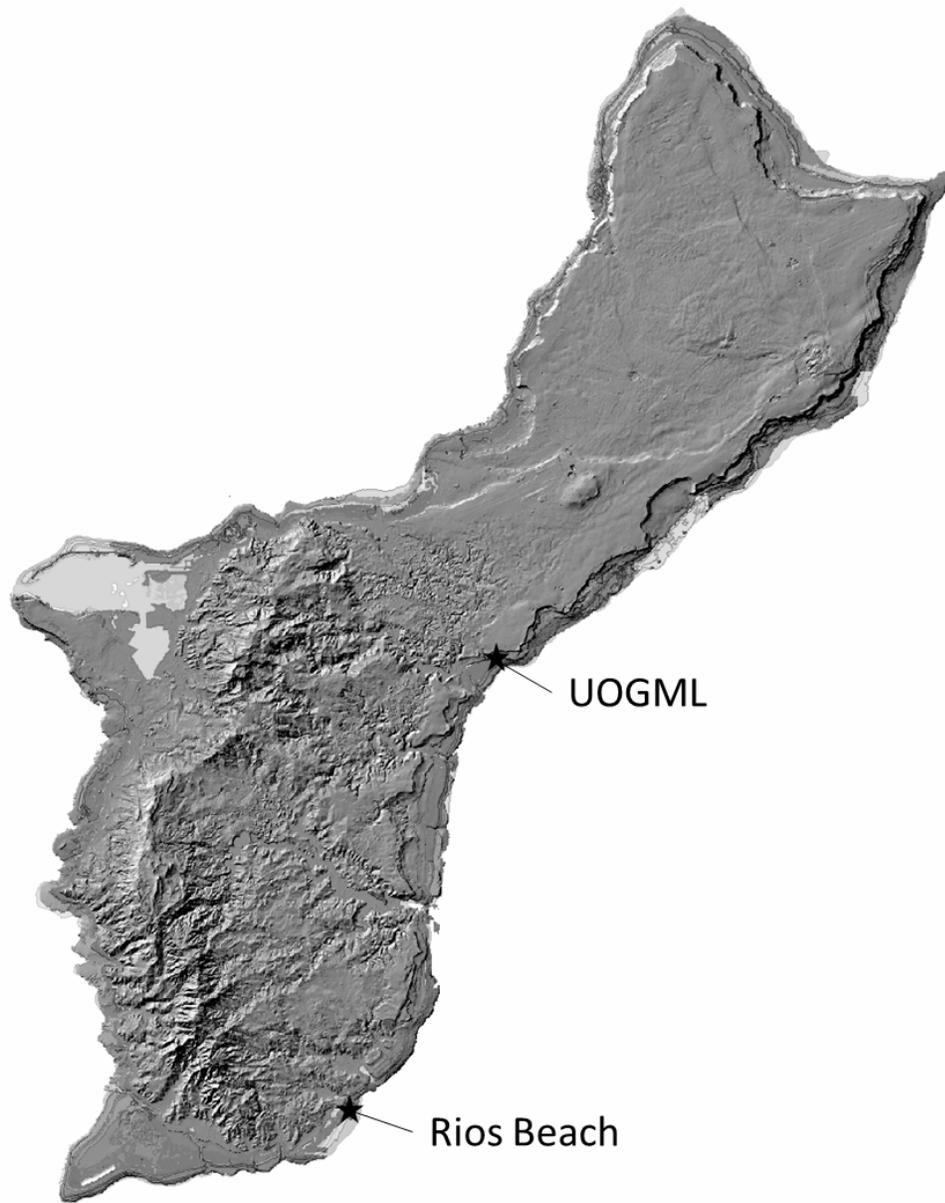
Figure 1: Location of the sampling site, Rios Beach, in southern Guam. Following night collections from Rios Beach, wild-caught *S. spinus* adults were transported to the University of Guam Marine Laboratory (UOGML).

S. spinus **Acclimation:**

Wild-caught fish were allowed to acclimate themselves to the UOGML seawater system for 14-28 days before initiating the experiments. During this time, fish were also metabolically "flushed" by feeding them with commercial fish food pellets, *ad libitum* (Emborski et al., 2012). Cement blocks were placed in all tanks used in this study so that fish could hide comfortably (Figure 2).

Figure 2: Wild-caught *S. spinus* (scribbled rabbitfish) adult acclimating in an aquarium tank at the UOGML.

Induction Experimental Design:

Due to limited tank space, an experimental trial consisted of seven (7) treatments conducted on a single day (n=1). Trials were then repeated five times, for a total sample size of five (n=5) per treatment, with days becoming a random variable. Twenty-four hours prior to beginning a trial, seven individual fish were weighed then allowed to acclimate to the treatment tanks by transferring each from the main holding tank to an independently-supplied 90-L flow-through tank supplemented with air (Figure 3). To reduce thermal and photo-stress during this study, the 90-L experimental tanks were shaded with mesh cloth (Figure 6) (Portz et al., 2006; Emborski et al., 2012; Harley, 2003). It is important to note that once transferred to a treatment tank, fish were no longer fed as trials began immediately after a final 24-hour acclimation period.
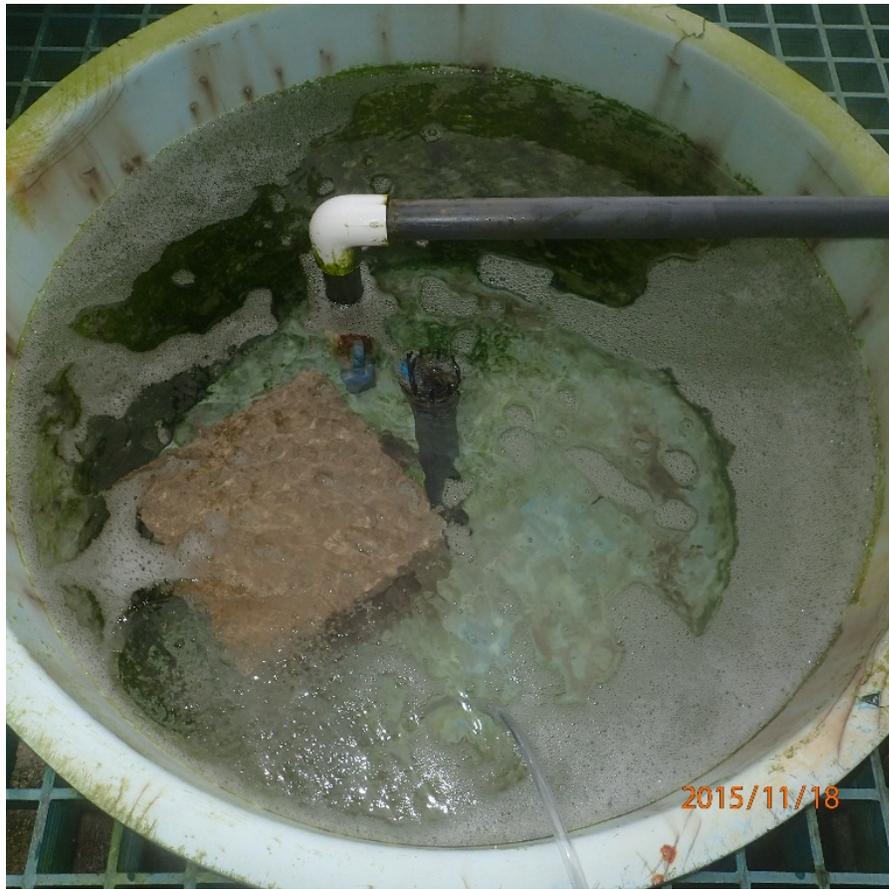
Figure 3: The tank set-up at the UOGML, supplied with a cement block, shade cloth, air, and fresh seawater from the flow-through system.

After the 24-hour acclimation period, the seven treatment groups were randomly distributed among the 90-L tanks (Figure 4). The intraperitoneal (IP) treatment tanks were labeled IP control (n=5) – soybean oil, IP EE2 (n=5) – 17α-ethinylestradiol in soybean oil, and IP BNF (n=5) – β-napthoflavone in soybean oil. In-water (IW) exposure tanks were labeled IW control (n=5) – ethanol, IW EE2 (n=5) – 17α-ethinylestradiol in ethanol, and IW BNF (n=5) – β-napthoflavone in ethanol, respectively. The seventh, "baseline group," consisted of fish unexposed to the delivery vehicles (soybean oil or ethanol) or xenobiotics. Instead, the baseline group was used to test for effects from the IP injection and/or IW exposure. Doses for the IP injections (mg/kg) were calculated using individual fish weights (see below; Table 1).
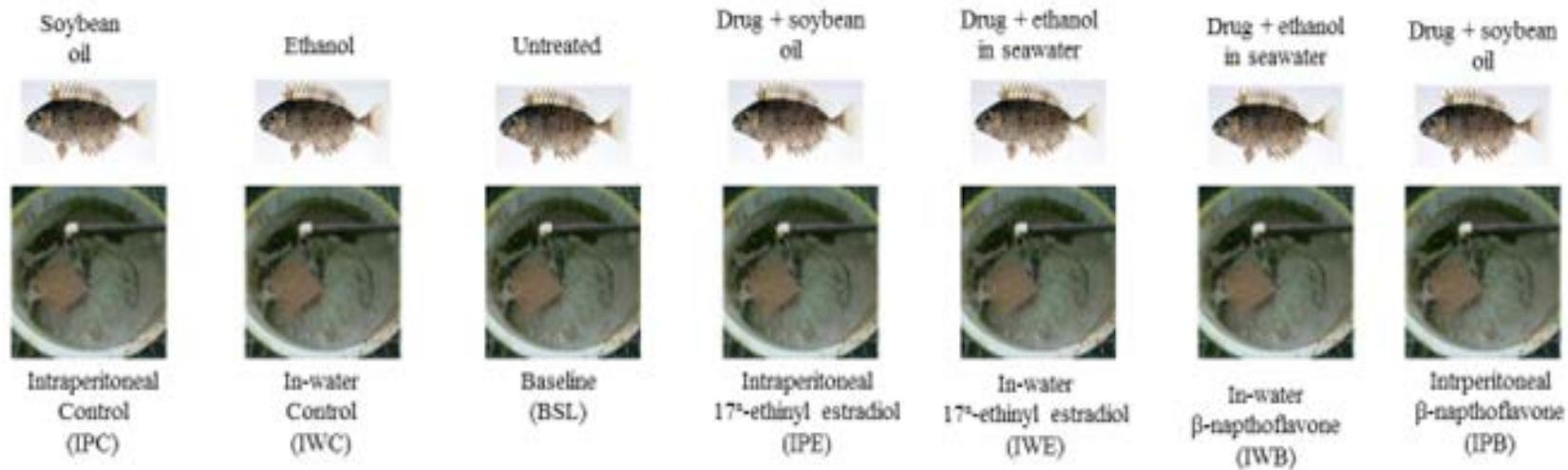
Figure 4: The induction experimental design for the in-water and intraperitoneal administration routes. Treatment groups were administered a single, 24-hour exposure.

Table 1: Attributes of *S. spinus* adults used in the experiments. Individuals that were either difficult to sex or lacking gonads were labeled "U" (undetermined).

Trial	Fish ID	Treatment	FL (cm)	BM (g)	Sex	Liver (g)	Notes
1	IPC 1	Intraperitoneal Control (SBO)	12.5	35.3	M	0.344	gravid
1	IWC 1	In-water Contol (etoh)	12.4	33.8	F	0.477	gravid
1	BSL 1	baseline	13	41.5	U	0.301	U
1	IPE 1	Intraperitoneal EE2	12.5	33.7	M	0.439	gravid
1	IWE 1	In-water EE2	12.6	37.3	F	0.343	gravid
1	IWB 1	In-water BNF	12.2	29.6	F	0.383	gravid
1	IPB 1	Intraperitoneal BNF	13.7	44.3	F	0.528	gravid
2	IPC 2	Intraperitoneal Control (SBO)	12	30.3	F	0.322	gravid
2	IWC 2	In-water Contol (etoh)	12	32.4	F	0.370	gravid
2	BSL 2	baseline	12.5	35.6	F	0.433	gravid
2	IPE 2	Intraperitoneal EE2	12.4	34.9	M	0.627	gravid
2	IWE 2	In-water EE2	12.1	32.1	F	0.923	gravid
2	IWB 2	In-water BNF	12.4	35.3	F	0.519	gravid
2	IPB 2	Intraperitoneal BNF	12.5	36.0	F	0.600	gravid
3	IPC 3	Intraperitoneal Control (SBO)	13.6	43.8	U	0.770	U
3	IWC 3	In-water Contol (etoh)	12.6	40.3	M	0.984	gravid
3	BSL 3	baseline	12.4	33.9	M	0.909	gravid
3	IPE 3	Intraperitoneal EE2	13.6	46.1	U	1.055	U
3	IWE 3	In-water EE2	12.5	38.9	M	0.977	gravid
3	IWB 3	In-water BNF	13.1	44.0	M	0.917	gravid
3	IPB 3	Intraperitoneal BNF	13.4	44.1	U	1.079	U
4	IPC 4	Intraperitoneal Control (SBO)	14.7	64.2	F	2.724	gravid
4	IWC 4	In-water Contol (etoh)	14.9	71.0	F	2.927	gravid
4	BSL 4	baseline	15	79.1	F	2.885	gravid
4	IPE 4	Intraperitoneal EE2	14.5	62.2	F	2.105	gravid
4	IWE 4	In-water EE2	15.1	71.0	M	1.079	gravid
4	IWB 4	In-water BNF	14.8	65.7	F	1.856	gravid
4	IPB 4	Intraperitoneal BNF	15.2	73.8	F	1.009	gravid
5	IPC 5	Intraperitoneal Control (SBO)	12.3	34.1	M	0.404	gravid
5	IWC 5	In-water Contol (etoh)	12.6	36.2	U	0.525	gravid
5	BSL 5	baseline	12.5	36.5	M	0.549	gravid
5	IPE 5	Intraperitoneal EE2	11.3	25.3	U	0.688	U
5	IWE 5	In-water EE2	12	31.0	M	0.709	gravid
5	IWB 5	In-water BNF	12	33.2	M	0.859	gravid
5	IPB 5	Intraperitoneal BNF	12.7	37.4	M	0.783	gravid

The UOGML flow-through seawater system was turned off during all trials to allow for a constant 24-hour exposure to β-napthoflavone (BNF) and 17α-ethinylestradiol (EE2), respectively (Kidd et al., 2007; Lange et al., 2001; Parrott and Blunt, 2005; Orn et al., 2003). Fish were euthanized after this 24-hour period, which has been shown sufficient for *S. spinus* CYP1A induction (Emborski et al., 2012). Again, these experimental trials were repeated a total of five times (n=5) with day as a random factor.

Routes of Exposure:

The present thesis expands on previous work by Emborski et al. (2012), who used a single intraperitoneal injection of BNF (solubilized in soybean oil) to induce *S. spinus* hepatic CYP1A. To be consistent, the IP exposure route was included within this study and consisted of a single intraperitoneal injection of BNF or EE2 solubilized in soybean oil. The IW exposure route, however, consisted of a single exposure of BNF or EE2 solubilized in ethanol. In other words, either soybean oil or ethanol served as delivery vehicles to administer the compounds.

The IP treatment dosages were approximately 50mg/kg of body weight (Emborski et al., 2012). EE2 and BNF were solubilized separately in soybean oil using sonication. Fish were injected approximately 0.5cm distal to the left pectoral fin (side view) using a 1-mL Becton-Dickinson insulin syringe. By inserting the syringe proximally 15° parallel to the body of the fish and approximately 0.5cm into the body cavity, rupturing the gallbladder could be avoided because bile acids have been demonstrated to inhibit hepatic monooxygenase activity (Nilsen et al*.,* 1998; Sinal and Bend, 1997). Immediately after injection, the flow-through was turned off and the time was recorded on water-resistant cards anchored beneath the tanks.

The IW treatment groups, were exposed to 6mg/L of BNF and EE2, respectively (Parrott and Blunt, 2005; Harrison, 2008). Similar to the IP exposure treatments, EE2 and BNF were solubilized separately in 1mL of ethanol, added to 1000-mL Erlenmeyer flasks containing fresh seawater from each tank, stirred then emptied back into the corresponding tank with the flow-through system turned off. This method of in-water delivery not only minimized overall use of the test compounds, but also prevented them from draining out of the tank and entering the UOGML effluent. Again, the exposure time was recorded on the cards beneath the tanks.

Water Temperature:

Because temperature can exhibit effects on metabolic activity (Figure 5), water temperature was measured using a glass total immersion Mercury thermometer. To calibrate the thermometer to the UOGML flow-through seawater system, it was placed in a flow-through tank, other than the treatment tank, for 72-hours prior to use. Particular attention was taken to ensure that the Mercury in the thermometer was submersed in the flow-through tank by placing a cable tie at the 80C mark. The thermometer was then placed into a 5cm^2 x 0.75cm (height) Styrofoam™ block which allowed the thermometer to float freely about the flow-through tank, while also being held in place by the cable tie. The water temperature of each treatment tank was recorded every six hours, and at the end of the exposure time just prior to dissection, by removing the thermometer from the flow-through tank and placing it in the treatment tank. After 5 minutes in the treatment tank, the thermometer and the Styrofoam™ block were lifted enough to see the Mercury, while leaving the bulb of the thermometer submerged. At eye level, the temperature was recorded.

During all trials, the tanks were covered with a transparent sheet to prevent precipitates or foreign material from entering the tanks (Figure 6). After the 24-hour exposure, the seawater effluent in each tank was removed and disposed into the local sewage wastewater system. Once emptied, tank treatment labels were removed, the tanks were cleaned, and the flow-through seawater restored. This entire experimental procedure was repeated for trials 2-5, after waiting at least seven days to assure that no carryover of compounds occurred.
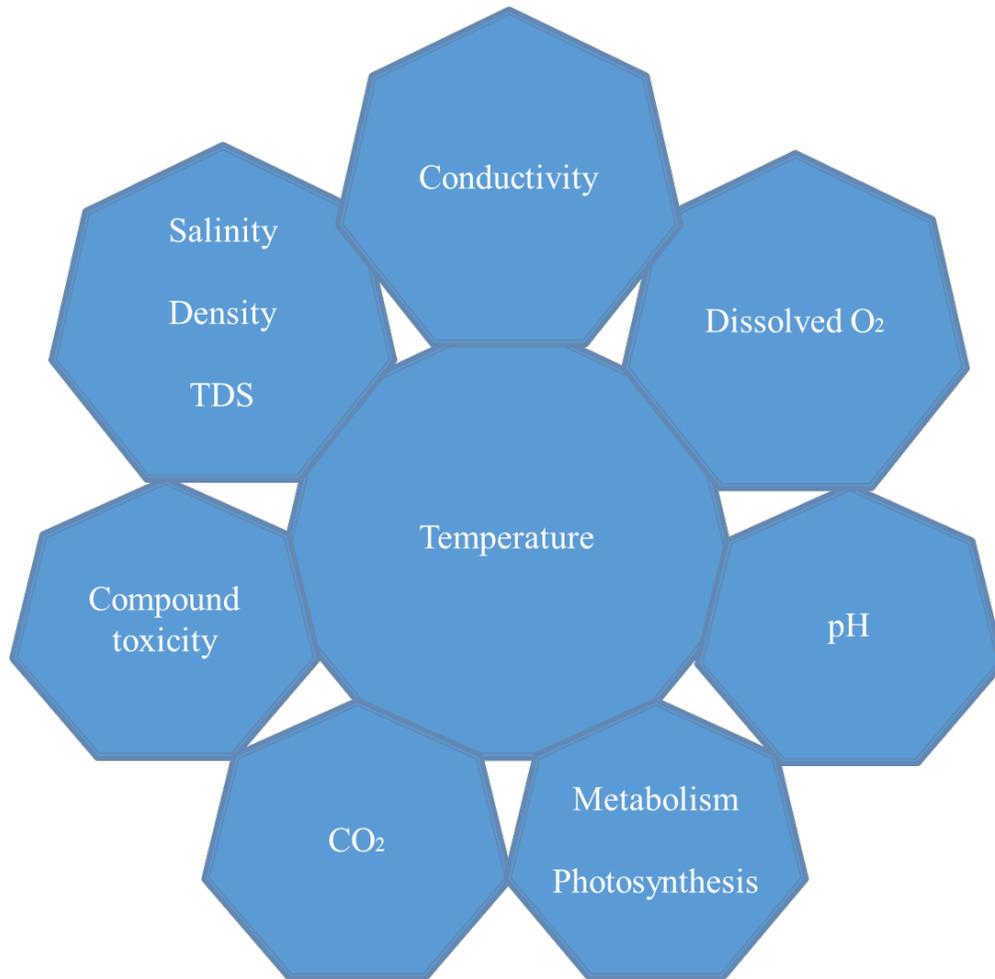
Figure 5: The effect of temperature on water quality.

Figure 6: After dosing, the tanks were covered with a transparent sheet to prevent precipitates or foreign objects from entering the tanks and experimental information was recorded on water-resistant cards anchored beneath the tanks.

S. spinus **Liver Dissections:**

For all dissections, stainless steel dissecting tools were sterilized by immersion in molecular-grade ethanol and flaming, and subsequently treated with RNaseZap® immediately prior to use (Rodrigo et al*.,* 2002; Morin et al., 2010; Narasimha et al., 2006). At the end of the 24-hour exposure, fish were euthanized by pithing, followed by decapitation – our institutionally approved method of euthanasia. Livers were dissected immediately, less than 60 seconds from the time of death. To do this, an incision parallel to the length of the body was made beginning approximately 2cm distal from the mouth and 1 cm from the base of the dorsal fin using a straight-edge stainless steel razor blade (see Figure 7). The area of the incision was approximately 4cm x 2cm, which was determined as adequate from preliminary dissections. Care was taken to ensure that the incision penetrated the flesh without rupturing organs, especially the stomach and gallbladder. The flesh was peeled away using forceps, exposing the organs, and enabling easy view and access to the liver (Figure 7). Livers were removed intact by carefully severing the connective tissue, followed by the hepatic artery then immediately immersed in ice-cold homogenization buffer (pH 7.4), blotted dry, weighed, wrapped in pre-labeled aluminum foil, and flash-frozen on dry ice before cryo-storage at -80C (Nilsen et al., 1998; Mutter et al., 2004; Kasahara et al., 2006; Florell et al., 2001; Collart and Oliviero, 2001; Leaver and Key, 1967).

Fork Length-Body Mass:

Fork length and body mass were measured to determine if such parameters could be used to determine gender of *S. spinus*. Fork length was measured using a fish board

with a measuring tape attached to the base of the board. Body mass was measured using a digital balance (capacity 1200 g x 0.1 g).

Individual attributes such as the presence/absence of gonads and liver mass were also recorded (Table 1). Individuals that were either difficult to sex or lacking gonads were labeled "undetermined".

Figure 7: *S. spinus* livers (in yellow). The area of the incision was approximately 4cm x 2cm.

S. spinus **Microsome Purification:**

Liver microsomal (S9) fractions were prepared using protocols from Nilsen et al, (1998) and the International Organization for Standardization (ISO; 2007) modified for this study. Using a Bead Bug® micro-tube homogenizer, individual livers were homogenized in ice-cold 0.1M sodium-phosphate buffer (pH 7.4) at a volume equal to four times the liver weight (e.g., 1g of liver tissue to 4mL buffer) (Nilsen et al., 1998). The resulting liver homogenates were used to create a post-mitochondrial supernatant (PMS). Further centrifugation (100,000 x g for 60 minutes at 4C) of the PMS produced an S9 pelleted fraction (Figure 8), which was resuspended in ice-cold 0.1M sodium-phosphate buffer with 20% glycerol (pH 7.4) (Nilsen et al., 1998).
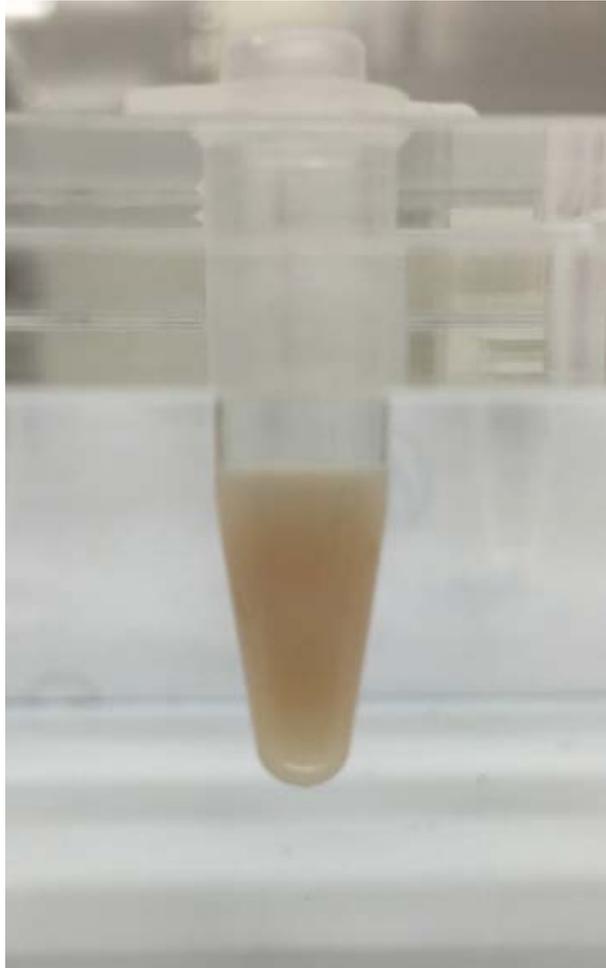
Figure 8: *S. spinus* hepatic microsomal (S9) fraction used to quantify total protein concentration and CYP-mediated metabolic activity.

For storage, the S9 fractions were preserved by dripping aliquots (ca. 100 µL) into liquid N_2. This method provided cryopreserved beads which were separated easily, and transferred while frozen which prevented sample degradation when performing individual assays. The beads were stored at -80C until needed for the total protein and enzyme activity assays.

S. spinus **Bradford Assay:**

Total protein concentration was quantified using the Bradford Assay. A reference protein was selected, which was as similar as possible to the protein under investigation, to perform the standard curve. Aliquots of the S9 fractions were thawed on ice and added to the Bradford reagent dye serial dilutions.

The microplate reaction mixtures were prepared according to the BioRad© quick-start Bradford protein assay manual. Total protein concentrations were calculated from a bovine-serum albumin standard curve (Figure 9) which was quantified using a Promega Glomax [®] multi-detection microplate reader (optical density = 600nm). For CYP-mediated assays, optimal total protein concentrations were observed at ~5-15 mg/mL.
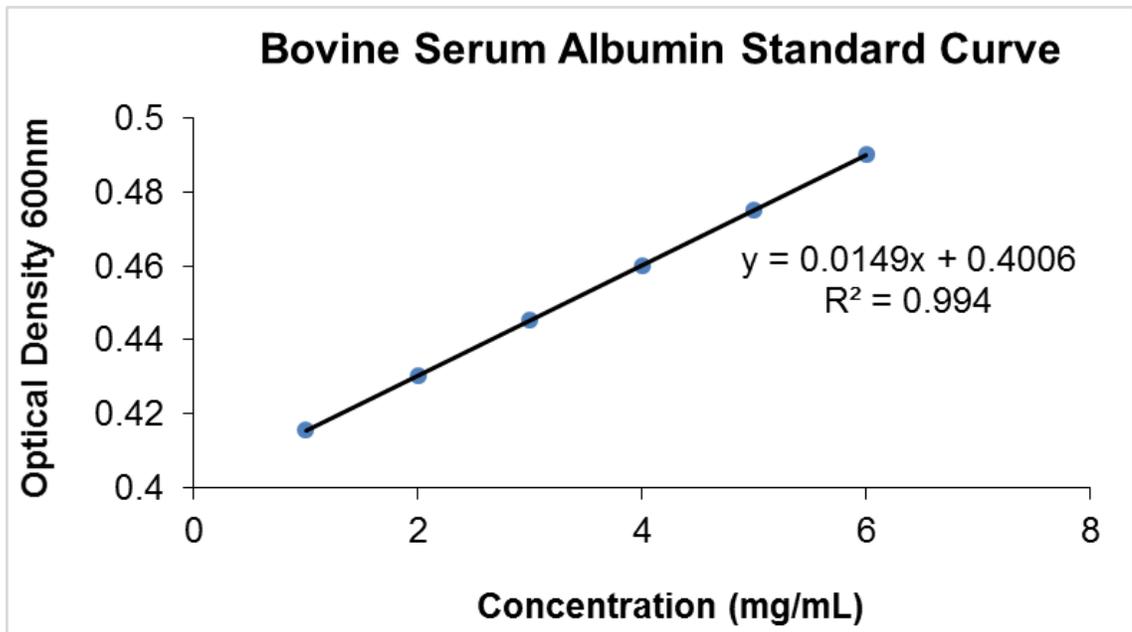
Figure 9: A bovine serum albumin standard curve used to quantify total protein concentrations as a function of optical density (R^2=0.994, 600nm).

S. spinus **CYP450-mediated Assays:**

CYP-mediated enzyme activity was quantified using the modified 7-ethoxyresorufin-O-deethylase (EROD) and 7-methoxyresorufin-O-deethylase (MROD) assays (Emborski et al., 2012; ISO, 2007; Nilsen et al., 1998). The optimum phosphate buffer solution pH, substrates (7-ethoxyresorufin and 7-methoxyresorufin), and cofactor (NADPH) were titrated prior to analyses (ISO, 2007; Nilsen et al., 1998). A resorufin standard curve (Figure 10) was generated each time the assays were conducted.

Reaction mixtures were assembled in separate wells of a 96-well microplate by serial addition of 200µL phosphate buffer solution (pH 7.8), 10µL alkoxyresorufin working solution (46µM), and 10µL S9 fraction (1-2mg/mL) to each well. The plate was then placed in a Promega Glomax® multi-detection microplate reader, which agitated the plate for 20 seconds using its shaker function (medium intensity) to allow reaction mixtures to homogenize. Immediately after mixing, 11.5µL NADPH (10mM) was added, and the plate shaken for another ten seconds to initiate the CYP-mediated catalytic cycle. CYP-catalyzed conversion of the respective alkoxyresorufin to resorufin was quantified fluorometrically (excitation at 530nm and emission at 590nm) at 27C ± 1C, every 60 seconds for a total of 15 minutes (15 measurements). Each sample was analyzed in duplicate.
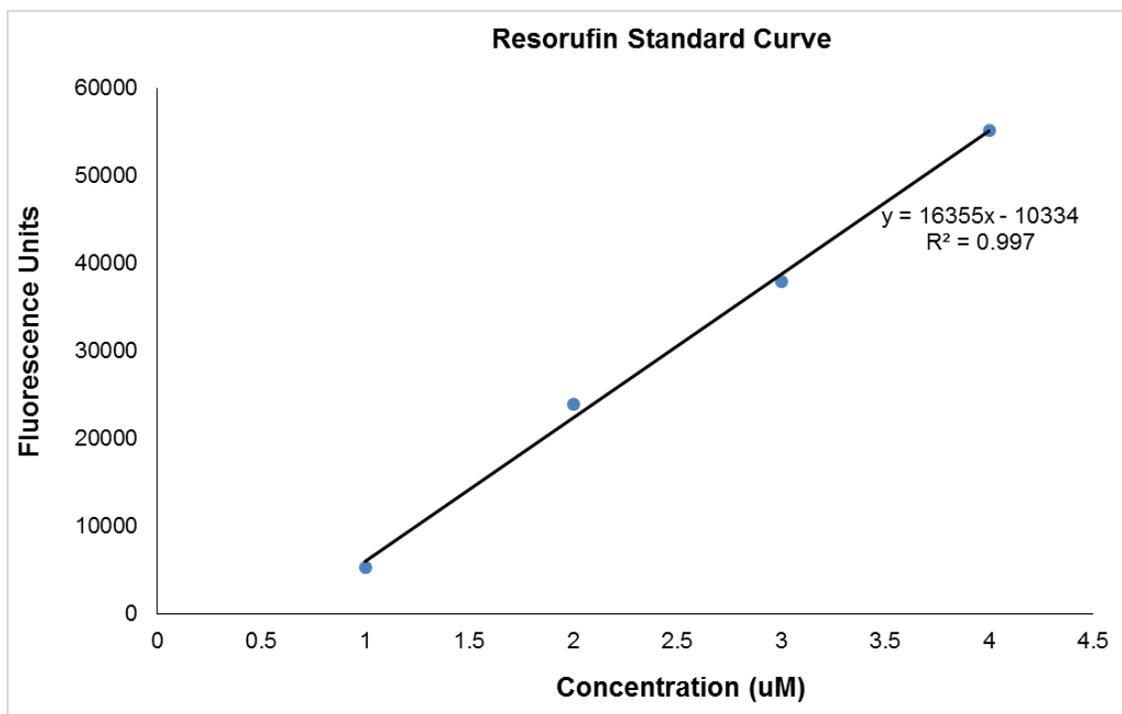
Figure 10*: A representative resorufin standard curve used to titrate the CYP-mediated reaction mixtures (R^2=0.997).

For each sample replicate, the mean time-dependent fluorescence increase (slope) was calculated at 15 consecutive points using linear regression. Resorufin equivalents were calculated by comparing individual slopes to their respective resorufin standard curve, enabling the overall CYP-mediated enzyme activity to be calculated for each sample. To account for potential microplate reader variability, samples were normalized to a positive control in the microplate (ISO, 2007; Nilsen et al., 1998). The arithmetic mean was calculated using measurements from replicate assays (ISO, 2007).

Statistical Analyses:

Due to a limited number of available tanks, experimental treatments were conducted on a number of different days (i.e., trials). Water temperature, therefore, was measured for each trial and tank to account for variations due to day or tank. Additionally, the treatment labels were randomized among the tanks for each trial to account for tank effects. Water temperature was measured every six hours and again at the end of the trial, totaling five recordings, which were partitioned by trial and tank. Trial and tank arithmetic means were calculated and plotted using histograms, with error bars reported as standard error from the mean.

Inferential analyses were conducted independently. First, the normality of the data was assessed using a Shapiro-Wilks test (Shapiro and Wilk, 1965). If the data were abnormal then they were transformed using the Box-Cox statistic (Box and Cox, 1964; Wessa, 2015) or log transformation. Second, the normalized data were analyzed using a Levene's test to assess variance homogeneity. Next, a one-way ANOVA assessed the differences between the trial and tank variances. Because water temperature variances were homogenous across trials and tanks, parametric inferential statistics were chosen.

Lastly, *post-hoc* analyses were conducted to determine whether or not differences between the trials and tanks were significant. This process was repeated for total protein concentration, and EROD and MROD activity, respectively. For *post-hoc* comparisons, treatments were grouped according to exposure route: controls (unexposed), intraperitoneal (IP), and in-water (IW), respectively.

Body masses versus fork length were analyzed using linear regression of scatter plots of *S. spinus* males and females. Body mass and fork length was assessed to determine which parameter could be used as an indicator of gender.

Results

Water Temperature:

Water temperature data were normal with homogeneous variances (Tables 2 and 3). The one-way ANOVA and *post-hoc* analyses indicated no significant differences in water temperature within the trials or tanks (Figures 11 and 12).

Table 2: Trial mean water temperature.

Trial	Mean Temperature (Celcius)	Standard Error
1	28	0.08
2	28	0.08
3	28	0.08
4	28	0.09
5	28	0.08
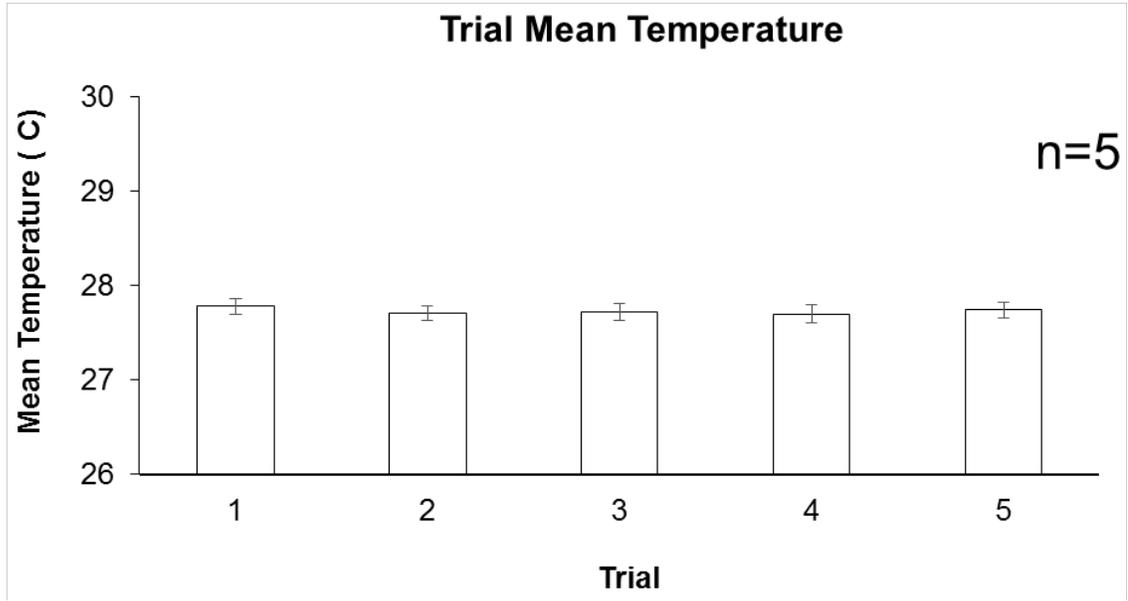
Figure 11: The trial mean water temperature, with error bars reported as standard error from the mean.

Table 3: Tank mean water temperature.

Tank	Mean Temperature (Celcius)	Standard Error
1	28	0.01
2	28	0.01
3	28	0.01
4	28	0.01
5	28	0.01
6	28	0.01
7	28	0.01
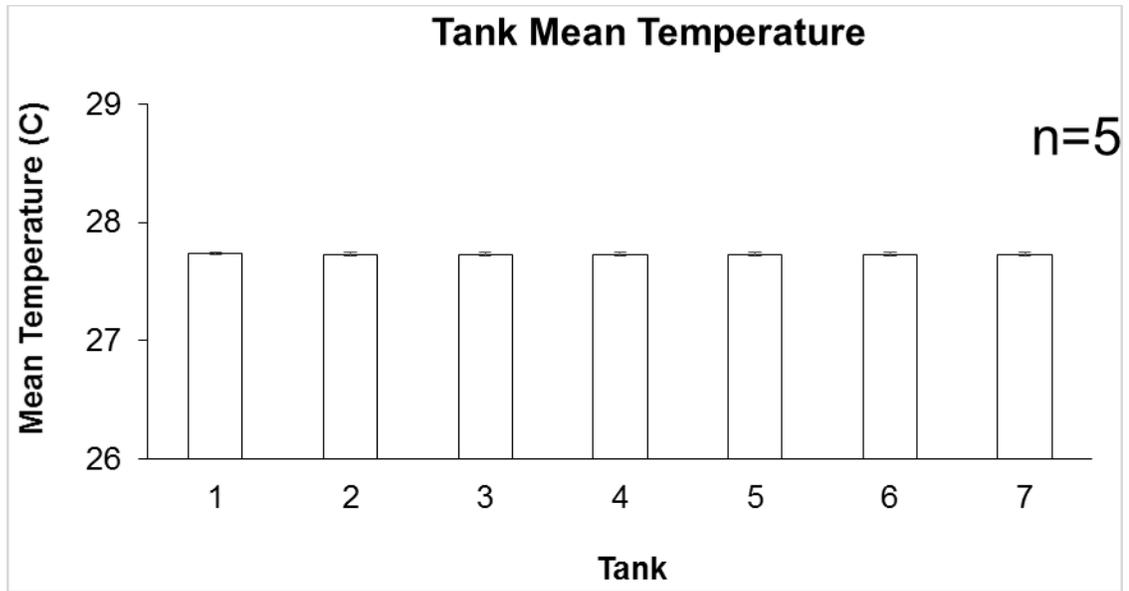
Figure 12: The tank mean water temperature, with error bars reported as standard error from the mean.

Total Protein Concentration:

Total protein concentration data were normal and homogeneous following a Box-Cox transformation (lambda = -2). Therefore, *post-hoc* t-tests assuming equal variances were chosen. Total protein concentrations in fish within the baseline, in-water, and intraperitoneal control groups were significantly higher compared to the in-water and intraperitoneal treatment groups exposed to either BNF or EE2 (Figure 13). In contrast, the intraperitoneal BNF and EE2 treatment groups had the lowest protein concentrations, indicating that exposure to these xenobiotics limited protein synthesis (Table 4). Overall, the in-water control treatment group had the highest, and the intraperitoneal BNF treatment group had the lowest mean total protein concentration (Table 4).

Table 4: Treatment mean total protein concentration.

Treatment	Mean Total Protein (mg/mL)	Standard Error
BSL	12.1	0.12
IPC	12.2	0.15
IWC	12.3	0.088
IPE	5.50	0.13
IWE	7.09	0.064
IWB	6.42	0.19
IPB	5.46	0.079
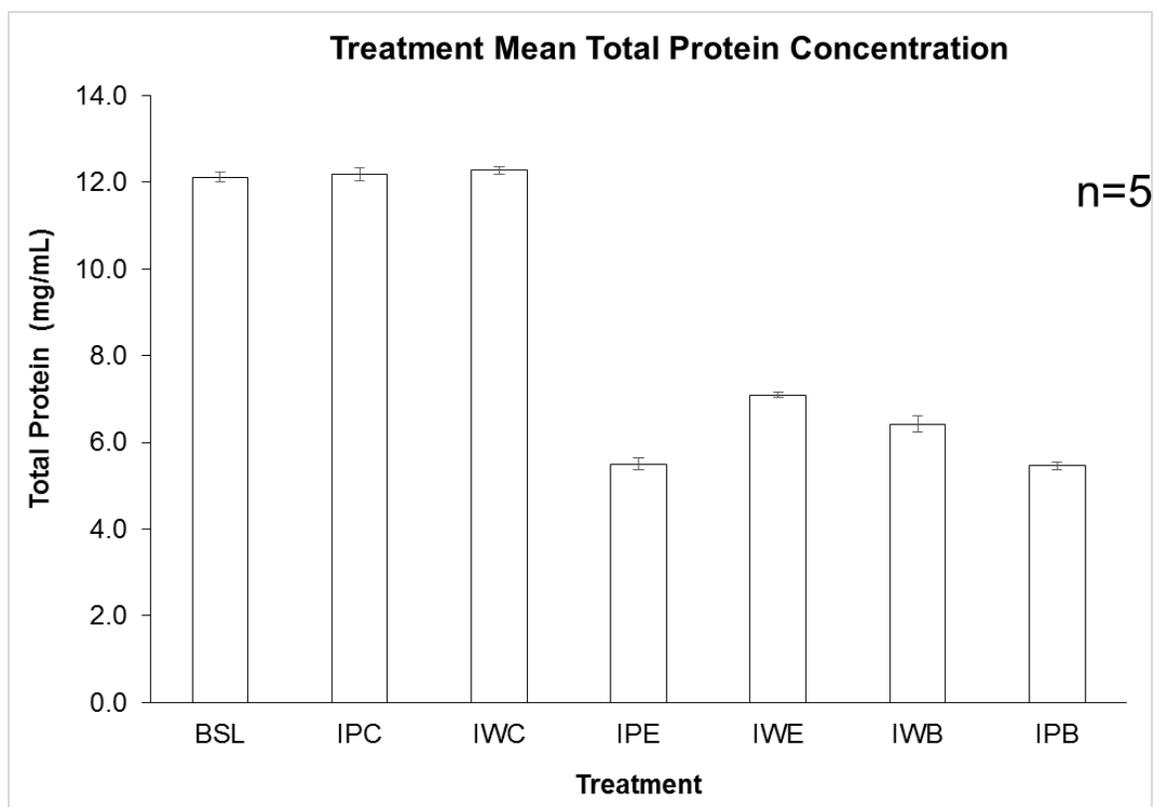
Figure 13: The mean total protein concentration of the *S. spinus* treatment groups, with error bars reported as standard error from the mean. The treatment groups are denoted: BSL - baseline, IPC - intraperitoneal control (soybean oil), IWC - in-water control (ethanol), IPB - intraperitoneal BNF, IPE – intraperitoneal EE2, IWB - in-water BNF, and IWE - in-water EE2.

ANOVA and *post-hoc* t-tests indicated no significant differences in total protein concentration between baseline (unexposed) and control (vehicle only) groups (Figure 14). However, *post-hoc* t-tests indicated significant differences (two-tailed) in total protein concentration between the intraperitoneal control vs intraperitoneal EE2 treatment groups - $t(8) = 29.35$, $p < 0.001$ (Figure 15); the intraperitoneal control vs intraperitoneal BNF treatment groups - $t(8) = 30.71$, $p < 0.001$ (Figure 15); the in-water control vs in-water EE2 treatment groups - $t(8) = 43.10$, $p < 0.001$ (Figure 16); and the in-water control vs in-water BNF treatment groups - $t(8) = 34.34$, $p < 0.001$ (Figure 16).
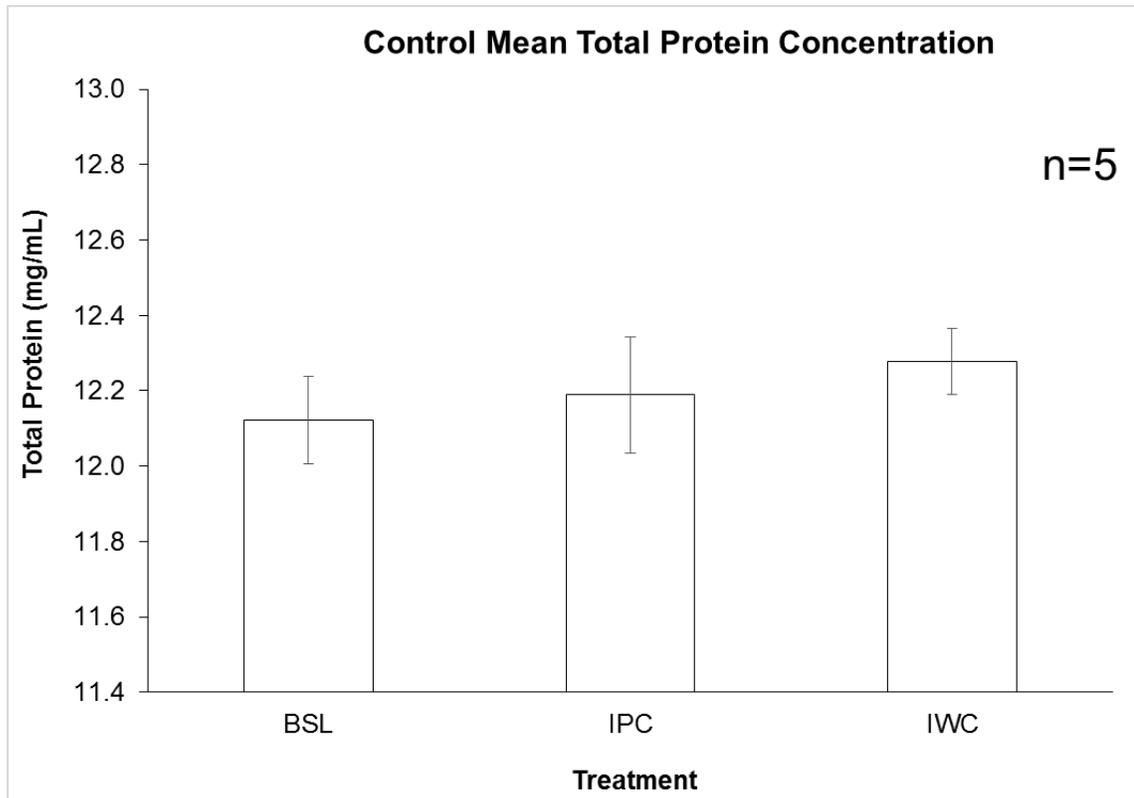
Figure 14: The graph (above) shows the mean total protein concentration of the control (unexposed) treatment groups, with error bars reported as standard error from the mean. Soybean oil and ethanol have no significant effects on *S. spinus* hepatic total protein concentration.

Figure 15: The graph (above) shows the mean total protein concentration of the intraperitoneal treatment groups, with error bars reported as standard error from the mean. Intraperitoneal administration of the xenobiotics significantly decreases *S. spinus* hepatic total protein concentration between (b) the intraperitoneal control vs (a) the intraperitoneal EE2 and BNF treatment groups.

Figure 16: The graph (above) shows the mean total protein concentration of the in-water treatment groups, with error bars reported as standard error from the mean. In-water administration of the xenobiotics significantly decreases *S. spinus* hepatic total protein concentration between (b) the in-water control vs (a) the in-water EE2 and BNF treatment groups.

EROD Activity:

EROD activity data were normal and homogeneous following a Box-Cox transformation (lambda = 1.27). Therefore, *post-hoc* t-tests assuming equal variances were chosen. EROD activity in fish within the baseline, in-water, and intraperitoneal control groups were significantly lower compared to the in-water and intraperitoneal treatment groups exposed to either BNF or EE2 (Figure 17). In contrast, the intraperitoneal BNF and EE2 treatment groups had the highest mean EROD activity, indicating that exposure to these xenobiotics increased CYP1A-mediated metabolism (Table 5). Overall, the intraperitoneal BNF treatment group had the highest, and the in-water control had the lowest mean EROD activity (Table 5).

Table 5: Treatment EROD activity means.

Treatment	Mean EROD (pmol/min/mg protein)	Standard Error
BSL	11.3	0.53
IPC	11.5	0.25
IWC	11.1	0.36
IPE	83.1	5.5
IPB	111	10
IWE	64.7	4.6
IWB	80.0	9.5

Figure 17: The mean hepatic EROD activity among experimental groups. The graph (above) shows the mean hepatic EROD activity, with error bars reported as standard error from the mean. The treatment groups are denoted: BSL - baseline, IPC - intraperitoneal control (soybean oil), IWC - in-water control (ethanol), IPB - intraperitoneal BNF, IPE - intraperitoneal EE2, IWB - in-water BNF, and IWE - in-water EE2.

ANOVA and *post-hoc* t-tests indicated no significant differences in EROD activity between the baseline and the control (unexposed) treatment groups (Figure 18). However, *post hoc* t-tests indicated significant differences (two-tailed) in EROD activity between each treatment and its respective control; i.e., the intraperitoneal control vs intraperitoneal EE2 treatment groups – $t(8) = 32.37$, $p < 0.001$ (Figure 19); the intraperitoneal control vs intraperitoneal β-napthoflavone treatment groups - $t(8) = 33.05$, $p < 0.001$ (Figure 19); the in-water control vs in-water EE2 treatment groups – $t(8) = 22.75$, $p < 0.001$ (Figure 20); and the in-water control vs in-water β-napthoflavone treatment groups – $t(8) = 22.97$, $p < 0.001$ (Figure 20).

Figure 18: The graph (above) shows the mean hepatic EROD activity of the control (unexposed), with error bars reported as standard error from the mean. Soybean oil and ethanol have no significant effects on *S. spinus* hepatic EROD activity.
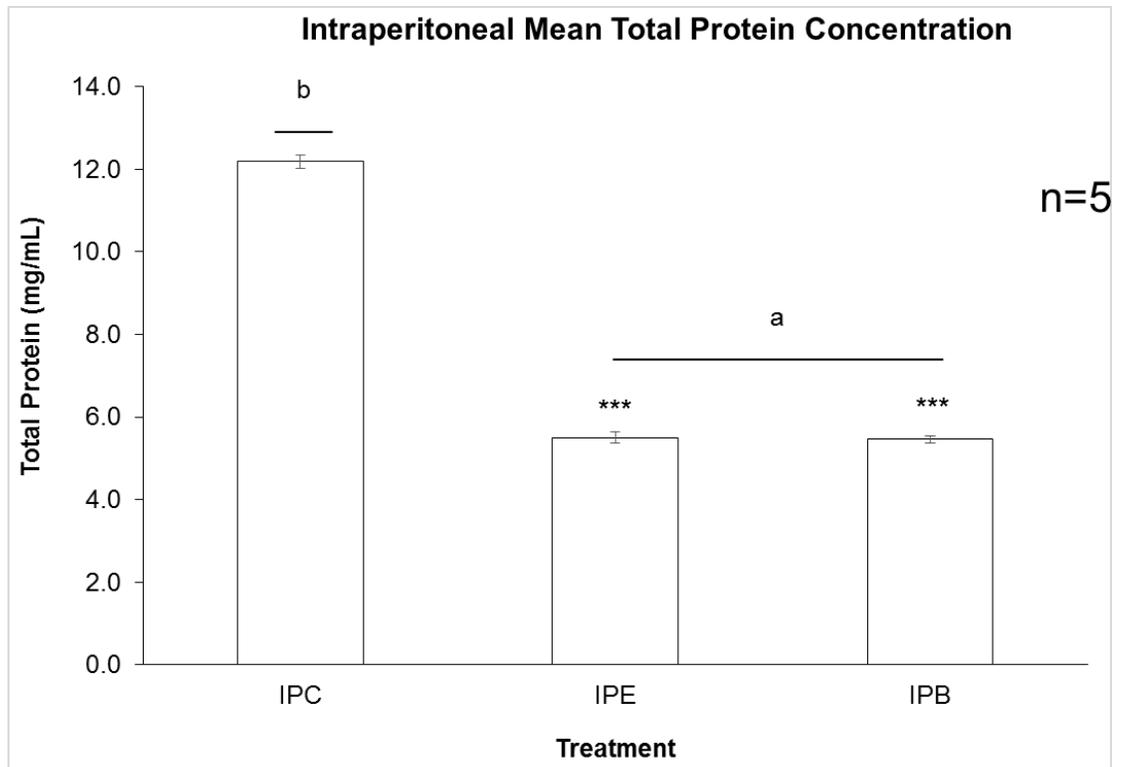
Figure 19: The graph (above) shows the mean EROD activity of the intraperitoneal treatment groups, with error bars reported as standard error from the mean. The intraperitoneal administration of BNF and EE2, respectively, significantly increases *S. spinus* hepatic EROD activity between (b) the intraperitoneal control vs (a) the intraperitoneal EE2 and BNF treatment groups.
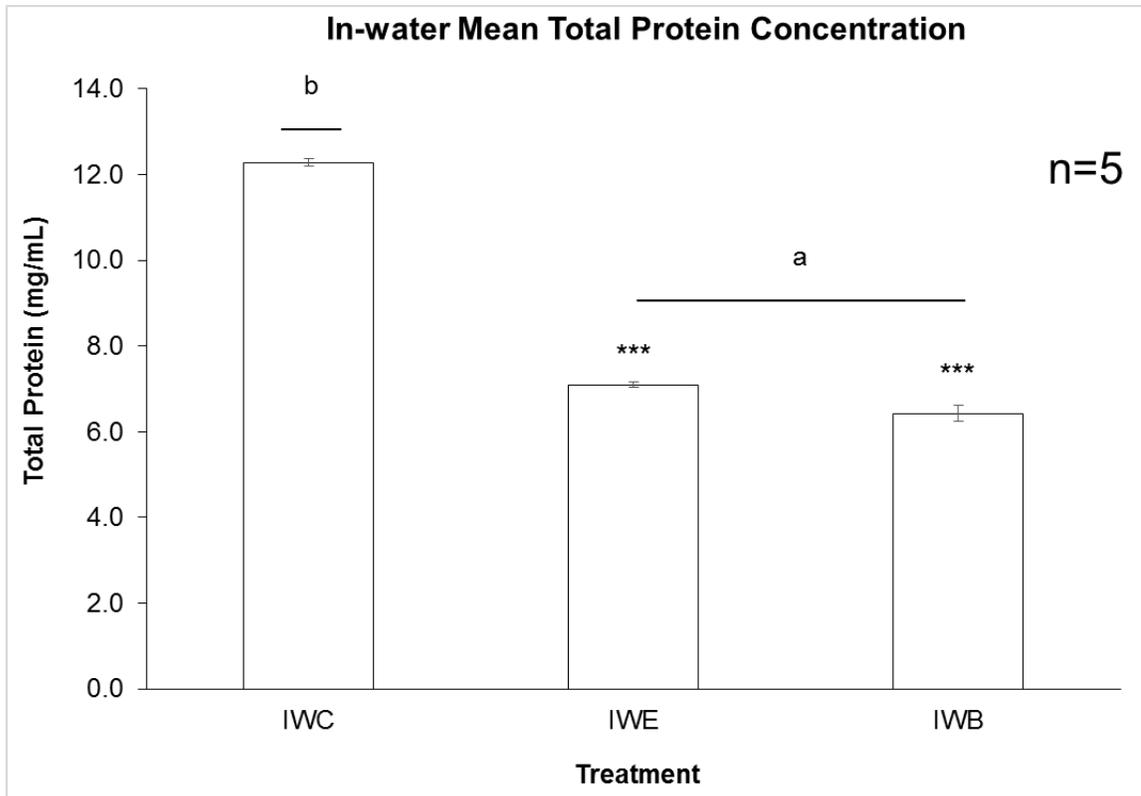
Figure 20: The graph (above) shows the mean EROD activity of the in-water treatment groups, with error bars reported as standard error from the mean. The in-water administration of BNF and EE2, respectively, significantly increases *S. spinus* hepatic EROD activity between (b) the in-water control vs (a) the in-water EE2 and BNF treatment groups.

MROD Activity:

MROD activity data were normal and homogeneous following a Box-Cox transformation (lambda = -2). Therefore, *post-hoc* t-tests assuming equal variances were chosen. MROD activity in fish within the baseline, in-water and intraperitoneal control, and in-water and intraperitoneal BNF groups were significantly lower compared to the in-water and intraperitoneal treatment groups exposed to EE2 (Figure 21). In contrast, the in-water and intraperitoneal EE2 treatment groups had the highest mean MROD activity, indicating that exposure to this xenobiotic increased CYP3A-meadiated metabolism (Table 6). Overall, the intraperitoneal EE2 treatment group had the highest, and the in-water control had the lowest mean MROD activity (Table 6).

Table 6: Treatment MROD activity means.

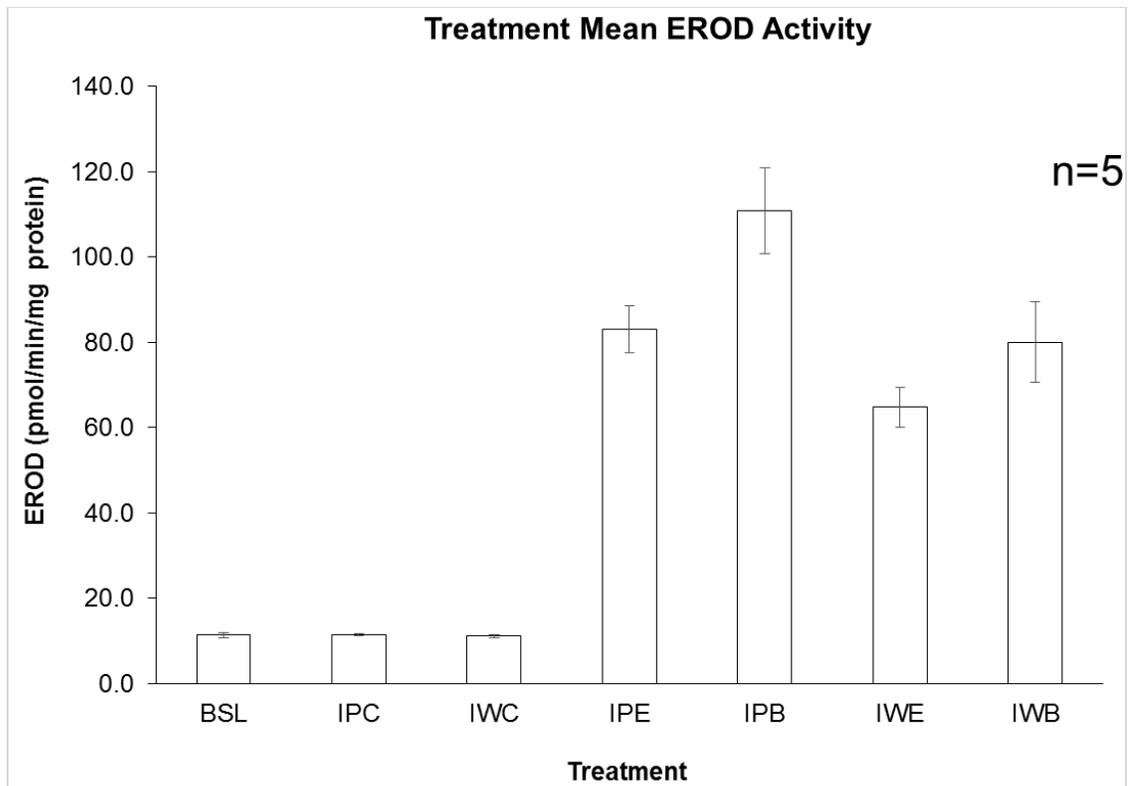| Treatment	Mean MROD (pmol/min/mg protein)	Standard Error
BSL	11.6	0.40
IPC	11.8	0.38
IWC	11.2	0.58
IPE	64.8	1.5
IWE	47.8	0.86
IWB	13.0	0.32
IPB	13.8	0.73
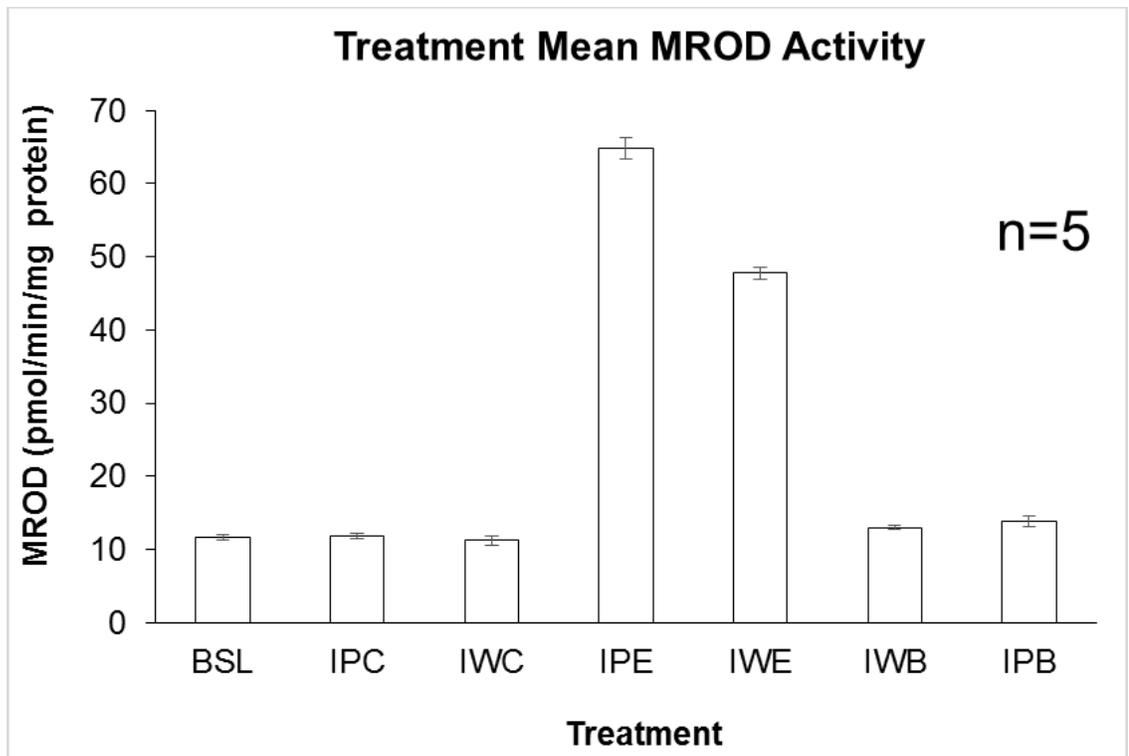
Figure 21: The mean hepatic MROD activity among experimental groups. The graph (above) shows the mean hepatic MROD activity, with error bars reported as standard error from the mean. The treatment groups are denoted: BSL - baseline, IPC - intraperitoneal control (soybean oil), IWC - in-water control (ethanol), IPB - intraperitoneal BNF, IPE - intraperitoneal EE2, IWB - in-water BNF, and IWE - in-water EE2.

ANOVA and *post-hoc* t-tests indicated no significant differences in MROD activity between the baseline and the control (unexposed) treatment groups (Figure 22). However, *post-hoc* t-tests indicated significant differences (two-tailed) in MROD activity between each treatment and its respective control; i.e., the intraperitoneal control vs intraperitoneal EE2 treatment groups – $t(8) = -21.7$, $p < 0.001$ (Figure 23); the intraperitoneal control vs intraperitoneal β-napthoflavone treatment groups - $t(8) = -2.48$, $p < 0.05$ (Figure 23); the in-water control vs in-water EE2 treatment groups – $t(8) = -26.2$, $p < 0.001$ (Figure 24); and the in-water control vs in-water β-napthoflavone treatment groups – $t(8) = -2.88$, $p < 0.05$ (Figure 24).
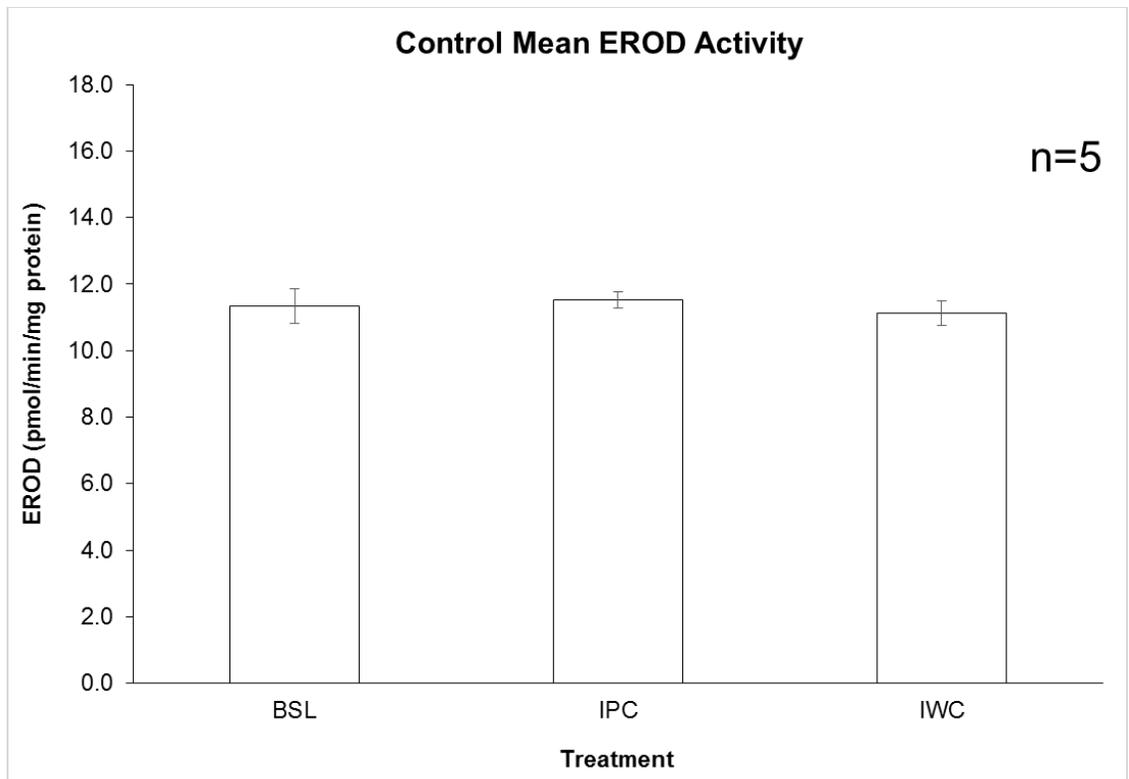
Control Mean MROD Activity

n=5
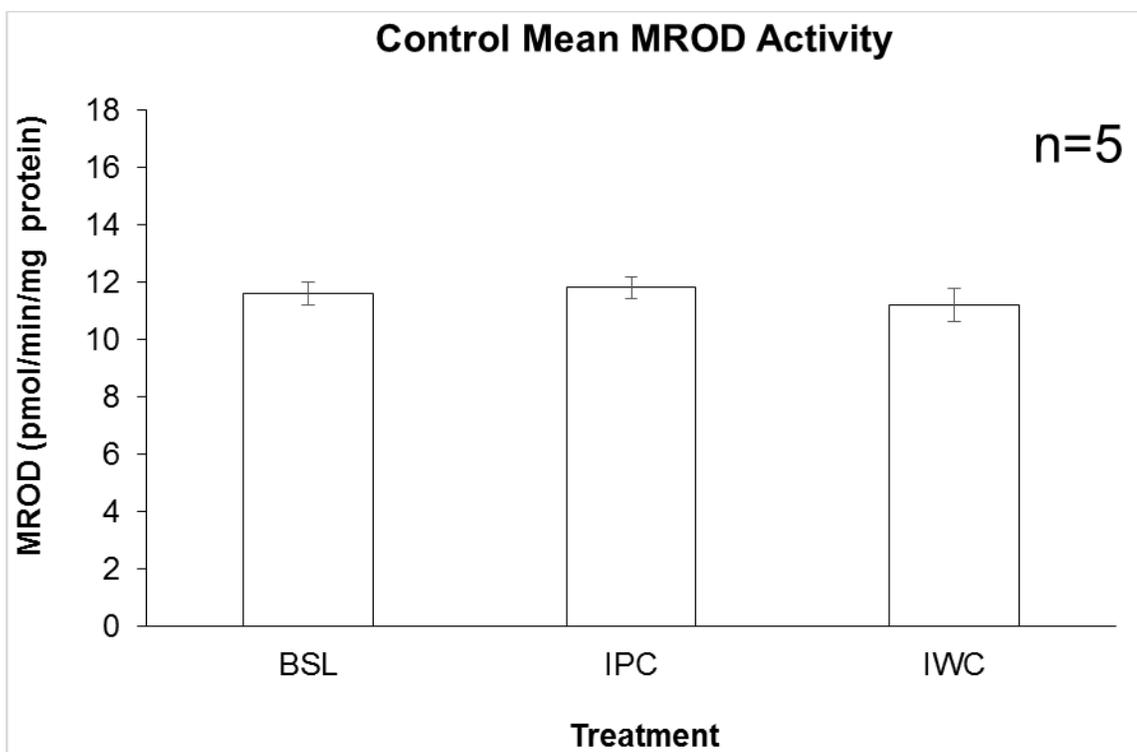
Figure 22: The graph (above) shows the mean hepatic MROD activity of the treatment groups unexposed to the drugs, with error bars reported as standard error from the mean. Soybean oil and ethanol have no significant effects on *S. spinus* hepatic MROD activity.
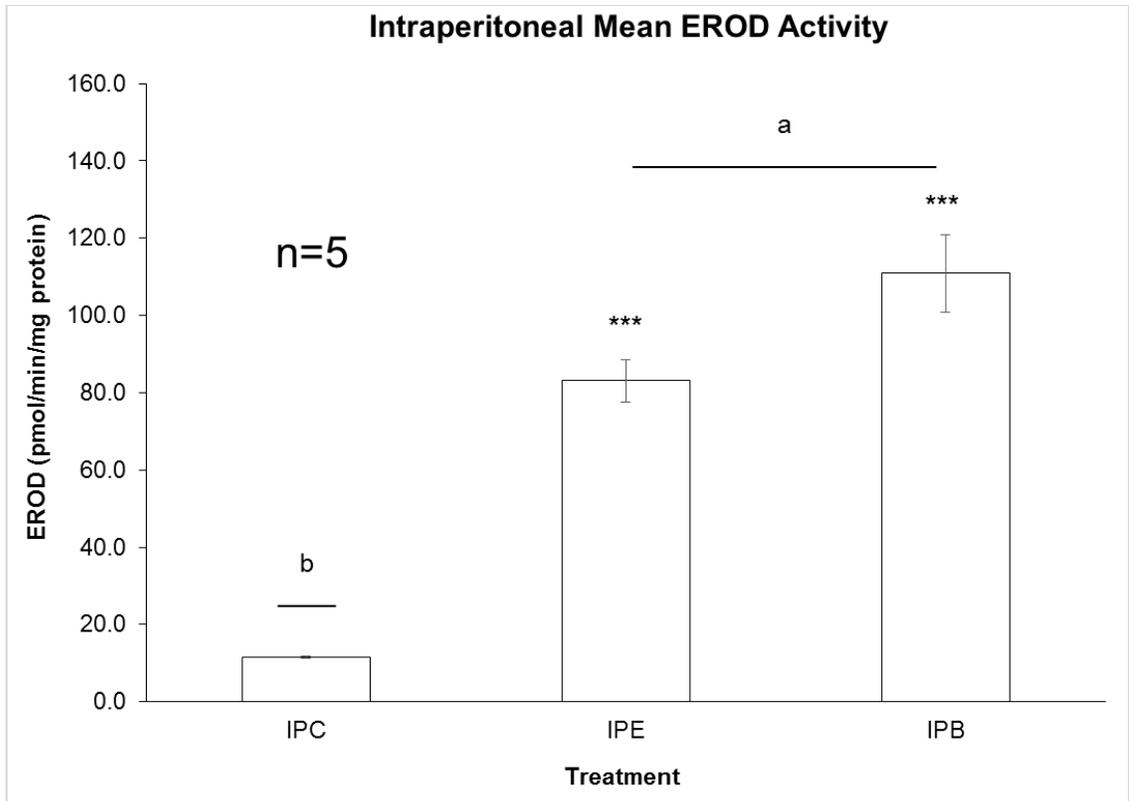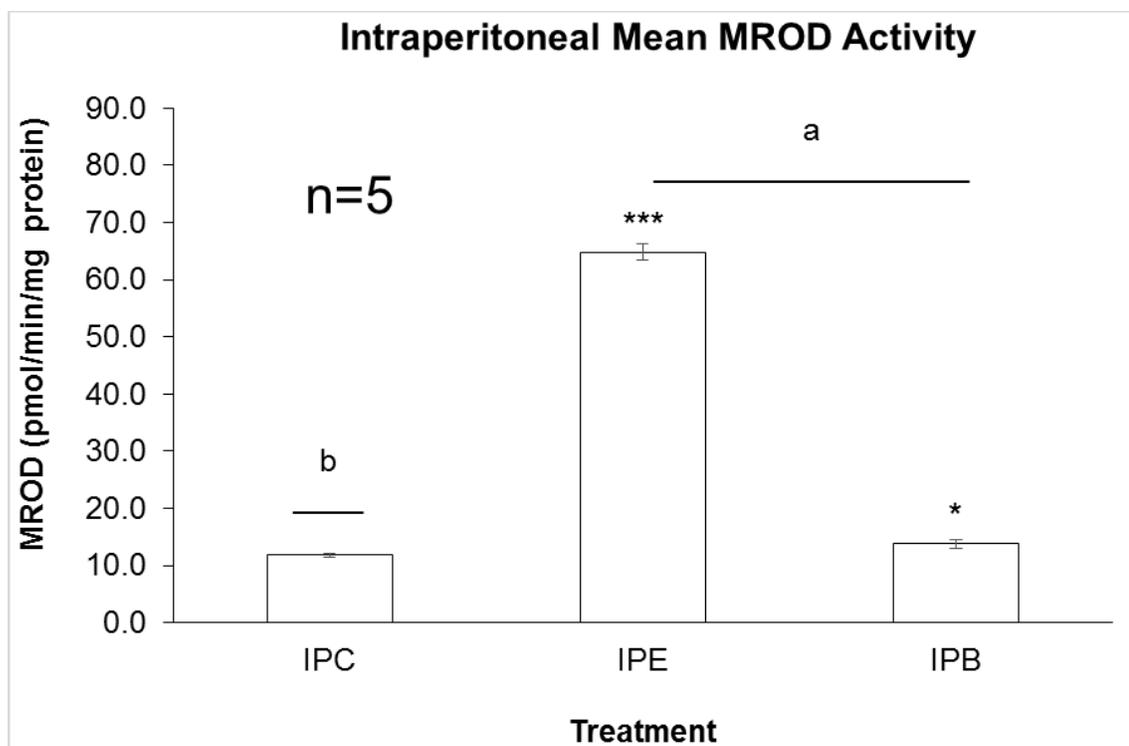
Figure 23: The graph (above) shows the mean MROD activity of the intraperitoneal treatment groups, with error bars reported as standard error from the mean. The intraperitoneal administration of BNF and EE2 significantly increases *S. spinus* hepatic MROD activity between (b) the intraperitoneal control vs (a) the intraperitoneal EE2 and BNF treatment groups.
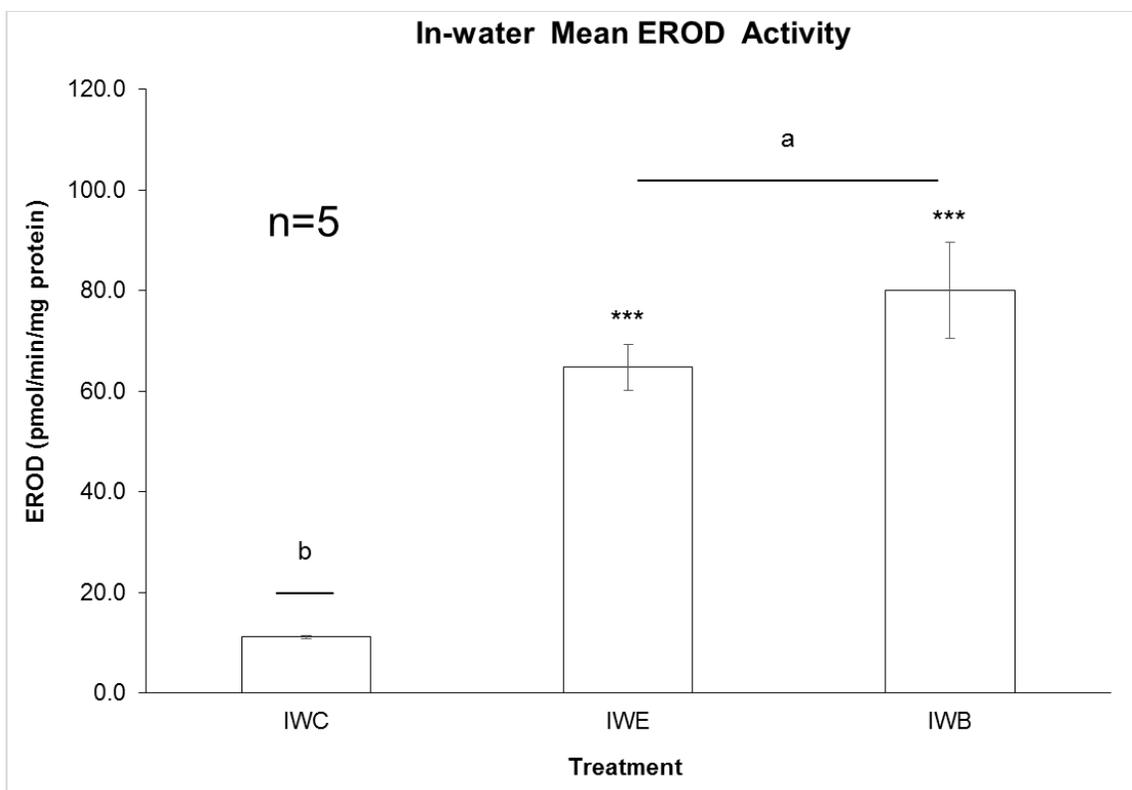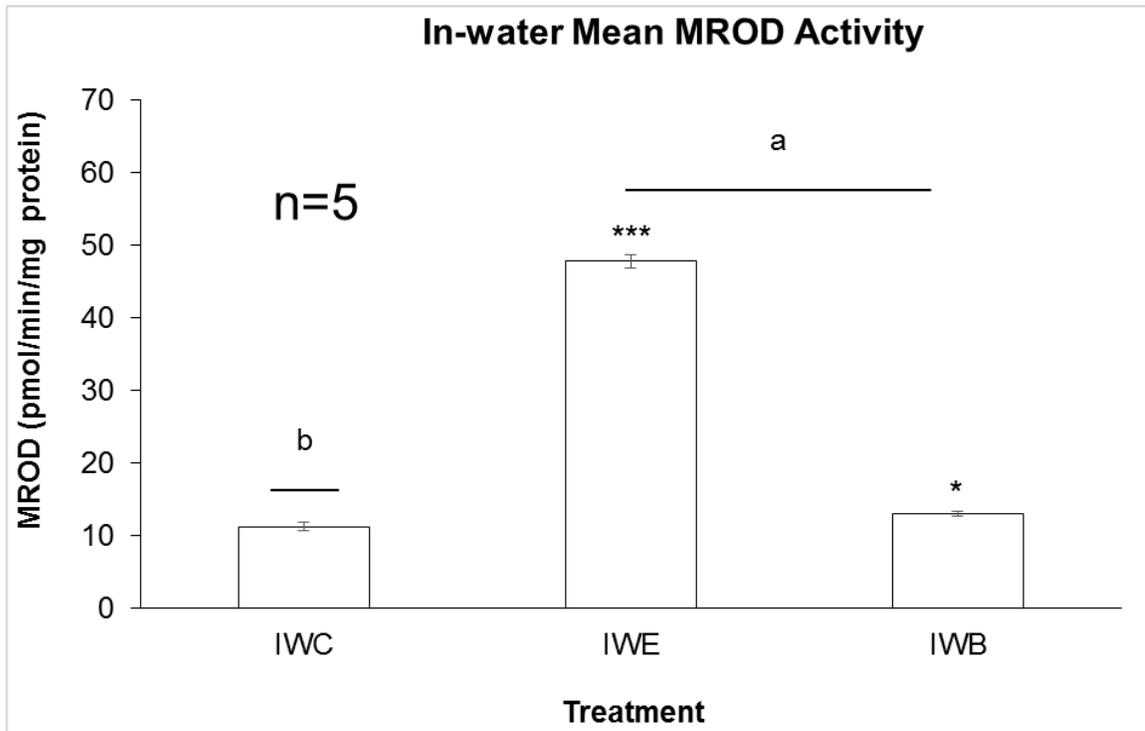
Figure 24: The graph (above) shows the mean MROD activity of the in-water treatment groups, with error bars reported as standard error from the mean. The in-water administration of BNF and EE2 significantly increases *S. spinus* hepatic MROD activity between (b) the in-water control vs (a) the in-water EE2 and BNF treatment groups.

Discussion

This study built upon Emborski et al. (2012), who focused on characterizing changes in CYP1A activity and protein level in response to a single intraperitoneal injection of BNF. This study further establishes *S. spinus* as a sentinel species for marine pollution by evaluating changes in the activity of two major hepatic enzymes (CYP1A and CYP3A) of *S. spinus* in response to being exposed to two different drugs (EE2 and BNF) under two different circumstances (IP-injection and passive absorption from the surrounding seawater). These changes were demonstrated by first assessing total protein concentration, and then CYP-mediated (EROD and MROD) activity among groups.

The Bradford assay allowed for the quantification of total protein concentration for *S. spinus* individuals. This differed from Emborski et al. (2012), however, who specifically characterized the relative abundance of CYP1A protein using an immuno-based assay (i.e., ELISA). Given the specificity of the ELISA results in Emborski et al. (2012), the increases in EROD activity in the present thesis can also be attributed to increased CYP1A protein (gene induction), rather than post-translational modification(s).

Total protein concentrations were compared according to their respective routes of exposure: controls (baseline, in-water, and intraperitoneal), intraperitoneal, and in-water. For the control (unexposed) groups, the in-water control group had the highest mean total protein concentrations, whereas the intraperitoneal β-naphthoflavone group had the lowest mean total protein concentration. The in-water control group might have had gravid females accounting for higher total protein concentration, as ovary ripening may influence total hepatic protein concentrations (Pagana and Pagana, 2010; Emborski et al., 2012). Gender was difficult to determine for *S. spinus* prior to euthanasia; sex

could only be definitively evaluated by examining reproductive organs. Those that were labeled "undetermined" were difficult, but subsequently grouped with the males. In total, there were more males (n=19) than females (n=16) used in the experiments which, again, is not easily controlled for in experiments utilizing *S. spinus*.

Fork length was not a useful indicator of sex. It was, however, a useful indicator of distinguishing between adults (~ 12 cm) and juveniles (< 12 cm) during field collections. Evidently, males and females can have similar fork lengths, but differ in body mass (unpublished data). Sex determination may become possible by considering *S. spinus* girth, determined by drawing a string around the fish at its widest point, marking where the string overlaps, and measuring the distance between the overlapping points on a conventional ruler; analogous to measuring the circumference of someone's waist (Florida Fish and Wildlife Conservation Commission, 2016). Although the *S. spinus* individuals were not measured this way, it seems logical that girth could play a role in assigning sex prior to enrolling wild-caught *S. spinus* in gender-specific studies. In fact, knowing the girth is important when trying to certify a sportfish for a record and provides useful information about a fish's relative condition (Florida Fish and Wildlife Conservation Commission, 2016). In contrast to fork length, body mass might better indicate sex because it can be resolved using girth. For the adult males, the shortest individual weighed the least and the longest individual weighed the most. This was not the case for adult females, however, as the shortest individual did not weigh the least and the heaviest individual did not weigh the most. Females, nonetheless, were aggregately larger; i.e., they commonly exhibited more girth than males. With this in mind, the "male" that weighed ~70g and had a fork length of ~15cm, could have been female. After

observing the size differences between the males and females, it is likely that this was the case. This may be important to control in future experiments as Emborski et al., (2012) had proposed that sexual differences could play a role in total protein concentration and EROD activity induction.

For the unexposed comparisons, the baseline, intraperitoneal and in-water vehicle controls showed no significant differences in total protein concentration, indicating that the controls (i.e., groups unexposed to the xenobiotics) were unaffected by administration of soybean oil, ethanol or route of exposure. This was consistent with Emborski et al. (2012), who also found no significant difference in CYP1A protein abundances between control (no injection) and vehicle control (single intraperitoneal injection of soybean oil) groups. For the intraperitoneal comparisons, the intraperitoneal control group had the highest mean total protein concentration, whereas the intraperitoneal β-napthoflavone group had the lowest. Comparisons between the control, and β-napthoflavone and 17α-ethinylestradiol treatment groups showed significant differences in total protein concentration. Based on these results, it seems logical that the presence of 17α-ethinylestradiol and β-napthoflavone in the body cavity of *S. spinus* lowers total hepatic protein concentration. Although previous studies have documented relative protein abundances being associated with β-napthoflavone exposure, it is intriguing that attenuation in total protein concentration was also found with 17α-ethinylestradiol. For the in-water comparisons, the control group had the highest mean total protein concentration, whereas the β-napthoflavone group had the lowest. Comparisons between in-water control, and in-water β-napthoflavone and 17α-ethinylestradiol treatment groups showed significant differences in total protein concentration. Similar to the

intraperitoneal groups, these results indicate that the presence of 17α-ethinylestradiol and β-napthoflavone in seawater lowers *S. spinus* total hepatic protein, and thus, liver function. Previous laboratory studies have not reported on total protein concentrations as a result of exposure to 17α-ethinylestradiol in tropical marine systems.

In humans, altered protein concentrations are indicators of adverse health effects. For example, most proteins in human serum are manufactured by the liver (Christensen, 2011), and a decrease in serum albumin is associated with lowered liver function, failure, and other diseases (Christensen, 2011). Because *S. spinus* exposed to β-napthoflavone and 17α-ethinylestradiol had attenuated protein concentrations, their livers may have been ailing. In contrast, elevated protein levels are associated with chronic inflammation, infection, and multiple myeloma (Christensen, 2011). However, all *S. spinus* were treated equally in experiments and compared accordingly, making it unlikely that the observed changes in total hepatic protein were coincidental. In fact, intraperitoneal and in-water controls showed no significant differences compared to the baseline group, indicating that *S. spinus* was unaffected by administration of the delivery vehicles. Although this may warrant further attention, the main objective of this thesis was to quantify change(s) in CYP-mediated activity rather than simply inferring a consequence to fish health.

For *S. spinus*, both the EROD and MROD assays show promise for drug-drug interactions at the metabolic level following an ecologically-relevant route of exposure; *e.g.*, passive absorption from the surrounding water. These findings resonate with those of Emborski et al. (2012), who characterized the CYP1A activity of *S. spinus* adults in the laboratory and at eight sites around the island of Guam. Clearly, these xenobiotics induce *S. spinus* CYP1A and CYP3A activity differentially, so drug-drug interactions

within this fish could be similar to those found in humans, and possibly even expand into the realm of interactions among naturally-occurring secondary metabolites.

The EROD activity was compared according to the respective routes of exposure: controls (baseline, in-water, and intraperitoneal), intraperitoneal, and in-water. It is important to note that all EROD activity calculations were normalized to total protein concentrations for the respective groups (ISO, 2007; Emborski et al., 2012). Among control groups, the intraperitoneal control group had the highest mean EROD activity, whereas the in-water control had the lowest mean EROD activity. The intraperitoneal control group might have had gravid females, accounting for aggregately lower mean EROD activity compared to the baseline (unexposed) group, as pregnancy has been shown to decrease EROD activity (Pagana and Pagana, 2010). Furthermore, the baseline (unexposed), and intraperitoneal and in-water vehicle controls showed no significant differences in EROD activity, indicating that *S. spinus* is neither affected by the concentrations of soybean oil and ethanol used in this study, nor by receiving an intraperitoneal injection. Again, these results resonate with laboratory experiments conducted by Emborski et al. (2012), who found no significant differences in EROD activity between the control (no injection) and the vehicle control (single intraperitoneal injection of soybean oil) groups. For the intraperitoneal comparisons, the β-napthoflavone group had the highest mean EROD activity, whereas the control group had the lowest. Comparisons between the intraperitoneal control, and intraperitoneal β-napthoflavone and 17α-ethinylestradiol groups showed significant differences in EROD activity. These results mirrored Emborski et al. (2012), who found significant differences in CYP1A activity between the intraperitoneal control and the intraperitoneal injection of β-

napthoflavone. Interestingly, in this study, the intraperitoneal 17α-ethinylestradiol treatment group had a lower mean EROD activity compared to the intraperitoneal β-napthoflavone treatment group, suggesting that the attenuation in the inductive response of CYPs can vary by sex, reproductive cycles, metabolic capacity, age, and/or the nature of the chemical (Emborski et al., 2012). The presence of 17α-ethinylestradiol and β-napthoflavone in the body cavity of *S. spinus*, nevertheless, significantly increases hepatic EROD activity resulting in a change in metabolic activity.

Interestingly, 17α-ethinylestradiol can also induce CYP1A activity is *S. spinus*, although the mechanism by which this occurs remains unresolved. This suggests that either CYP1A expression in *S. spinus* is influenced by additional nuclear receptors, such as the estrogen receptor, or the *S. spinus* Aryl Hydrocarbon Receptor is more promiscuous in substrate binding than its mammalian homologues. EE2 is also metabolized by human CYP1A and CYP2C9 (Wang et al., 2004), so these findings are not unusual. However, the major human enzyme to perform the 2-hydroxylation of EE2 is CYP3A4, and variation of this activity among humans accounts for the majority of differences in drug efficacy among individuals (Guengerich, 1990). Although 7-ethoxyresorufin may be metabolized by other *S. spinus* CYP isoforms, which would reduce its utility as a biomarker assay for this species, it is highly unlikely given that CYP3A is also expressed. These results, although preliminary, provide evidence for possible drug-drug interactions among CYP isoforms, which can influence the toxicity of a xenobiotic.

In conjunction with the EROD assay, the present thesis also observed changes in CYP3A activity using the MROD assay. In humans, the CYP3A isozyme is responsible

for 17α-ethinylestradiol metabolism, and its resulting metabolites have been characterized extensively (Wang et al., 2004; Li et al., 1999). As expected, increases in MROD activity were observed in both the intraperitoneal and in-water 17α-ethinylestradiol treatment groups. The intraperitoneal 17α-ethinylestradiol group had a higher mean MROD activity compared to the in-water group, which was also expected given the differences in dosage. Also similar to humans, increased CYP3A activity was not observed in β-napthoflavone groups which further suggests that much of the CYP1A and CYP3A induction pathways of *S. spinus* are similar to that of humans. Given that multiple human CYP isoforms work together to metabolize the plethora of xenobiotics that we encounter daily, it is quite possible that multiple CYP isoforms play similarly important roles in *S. spinus* and thus, human PCPs that enter marine environments may alter the metabolic fate of compounds within the diet of marine fishes.

In summary, xenobiotics play a role in the ecology of many organisms and their presence in ecosystems can help provide insight into their effects in biological systems. Previous research has shown several ways that manmade xenobiotics elicit biological effects within terrestrial, aquatic, and marine ecosystems. Xenobiotics can mimic naturally-occurring endogenous compounds, and in doing so are able to modulate physiological states that ultimately determine an organism's ability to maintain homeostasis. Xenobiotics can therefore change biological responses within wild organisms in ways that not only affect the fate of the xenobiotic, but also the compounds that organisms encounter naturally. Both mechanisms suggest that xenobiotics may induce drug-drug interactions with endogenous compounds and/or compounds with ecological relevance (e.g., feeding deterrents).

The inducibility of *S. spinus* CYP1A and CYP3A metabolic pathways can serve as a good model for demonstrating that PCPs within coastal environments affects the ecology of tropical marine ecosystems, suggesting that drug-drug interactions play a role in marine plant-herbivore ecology. Although it is too early to tell whether these affects are beneficial or detrimental given that ecological encounters often have a winner and a loser, it is safe to assume that both scenarios are true. Understanding, documenting, and monitoring the effects of exposure to xenobiotics is, therefore, one critical aspect of natural resource management that should continue to be addressed in tropical marine ecosystems throughout Micronesia.

Conclusion

Increasing evidence has indicated that diverse chemical compounds – specifically xenobiotics – in sewage effluent could be impacting coastal communities. Estrogenic activity was detected in Tanapag Lagoon, Saipan, Commonwealth of the Northern Marianas Islands (estrogen equivalence: 0.03 – 31.80ng/L) using the presence of bisphenol A (10.5 – 182ng/L) and estrone (0.05 – 0.1ng/L) from two sewage outfalls within the Lagoon (Harrison, 2008). Even though synthetic estrogens, such as 17α-ethinylestradiol, were characterized as xenobiotic pollutants decades ago, they remain prevalent in nearly all sewage treatment plant (STP) effluent, suggesting that ecosystems proximal to these sources may already be changing. Using *S. spinus* cytochrome P450s as an indicator of a xenobiotic's ability to elicit biological change(s), this study used the Bradford, and EROD and MROD assays to quantify change(s) in the laboratory effects of a known compound in STP effluent, 17α-ethinylestradiol, in response to ecologically-relevant routes of exposure; i.e., in the surrounding water. The present thesis

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demonstrates that, in the laboratory, *S. spinus* hepatic physiology changes after a single, 24-hour exposure to 17α-ethinylestradiol within the body cavity or the surrounding seawater. These results, although preliminary, strongly suggest that more work should be conducted to determine the implications of PPCPs being introduced into coastal marine environments and how these PPCPs adversely affect ecological interactions. With these results, we support management strategies to further address the effects of xenobiotic pollution in tropical marine ecosystems throughout Micronesia.

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