The Effects of Select Non-Steroidal Anti-Inflammatory Drugs on Green & Brown Hydra

by

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Abstract

Effluent pharmaceuticals are released at low concentrations into aquatic ecosystems through waste water treatment plants (WWTPs). Non-steroidal anti-inflammatory drugs (NSAIDs) are important contaminants in water cycles. In this study, several NSAIDs were assessed for their potential toxicity to *Hydra*. Chronic toxicity was evaluated using three NSAIDs on two different species of *Hydra*, *Hydra viridissima* and *Hydra littoralis*. Several negative correlations were observed between ibuprofen, naproxen, diclofenac toxicity and select endpoints in *Hydra.* However, these correlations generally occurred in only 25% of the 7 day exposure toxicity tests performed. Thus, environmentally relevant concentrations of ibuprofen, naproxen, and diclofenac did not have statistically significant chronic effects on either species of *Hydra* tested. Differences in endpoints between species were observed in only 25 to 50% of the experiments undertaken. Thus, current effluent concentrations of ibuprofen, naproxen and diclofenac are not likely deleteriously affecting this level of aquatic food webs receiving such contaminants.

Keywords: effluent, non-steroidal anti-inflammatory drugs (NSAIDs), *Hydra viridissima*, *Hydra littoralis*, chronic exposure, ibuprofen, naproxen, diclofenac

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Section 1: Introduction

1.1 Pharmaceuticals in Effluent

Waste water treatment plants (WWTPs) do not screen nor specifically filter xenobiotic substances out of effluent before it is released into the aquatic environment. Analysis of sewage samples from Baltimore Black River, USA, confirmed that a majority of personal care products were not removed through the wastewater treatment process (Yu *et al.*, 2006). As a result, effluent pharmaceuticals are continuously released at low concentrations into aquatic ecosystems through WWTPs (Brun *et al.,* 2006). Examples of effluent pharmaceuticals include cholesterol lowering agents (gemfibrozil & bezafibrate), analgesic drugs (ibuprofen & naproxen), anti-epileptic drugs (carbamazepine) and stimulants (caffeine) (Quinn *et al.,* 2008a). In a WWTP in India, ciprofloxacin was reported to be the most abundant effluent pharmaceutical found in literature (Larsson *et al.,* 2007). Ciprofloxacin was found at a concentration which exceeds toxic levels for specific strains of bacteria by over 1,000-fold (Larsson *et al.,* 2007). A large US study found that one or more effluent pharmaceuticals were present in 80% of the 139 streams sampled across the United States (Kolpin *et al.,* 2002). Other locations that pharmaceuticals have been found include drinking water, Atlantic Canadian surface waters, United Kingdom surface waters and waters in Taiwan (Hilton & Thomas, 2003; Brun *et al.,* 2006; Lin & Tsai, 2009; Ramirez *et al.,* 2009; Touraud *et al.,* 2011).

Pharmaceuticals can have endocrine disruption effects, cytostatic properties, genotoxic effects and chronic toxicity effects on non-target organisms. Endocrine disruption refers to the capacity to interfere with an organism's hormone system. One such effluent pharmaceutical is ethinylestradiol, which affects a whole host of important reproductive processes (Rahman *et al.,* 2009). Cytostatic properties allow effluent pharmaceuticals to inhibit cell growth and division,

and genotoxic effects result in DNA damage (Bakare *et al.,* 2009). Teratogenicity causing fetal malformations has been found in invertebrates after exposure to specific effluent pharmaceuticals (Johnson & Gabel, 1983). Changes in sex ratios have been reported in *Daphnia* exposed to these types of pharmaceuticals (Flaherty & Dodson, 2005). Also, changes in population dynamics can occur, for example recruitment in juveniles increased in *Gammarus pulex* exposed to effluent pharmaceuticals (Watts *et al.,* 2001). Aquatic biota experience lifelong exposures to low levels of such chemicals, and are thus more likely to have chronic rather than acute effects (Carlsson *et al.,* 2006). A decrease in an aquatic organism's fitness is one such chronic effect of exposure to these types of pollutants (Jones *et al.,* 2002). Pregnant women and children could also be at risk since children are considered to be 8 times more sensitive than adults (Collier, 2007). Effluent pharmaceuticals have such a diverse range of effects because they were originally designed to be biologically active via modification of physiological and/or biochemical functions. Also, these contaminants are continuously introduced into surface water (pseudo-persistent) leading to life-long exposure of aquatic biota (Pascoe *et al.,* 2003). Pharmaceuticals enter aquatic environments as complex mixtures, which could produce a total toxic effect that is greater than the individual toxic effects (synergism), increasing their toxicity. Quinn *et al.* (2009) found evidence of this, showing that the toxicity of individual effluent pharmaceuticals in a mixture was 2-3 orders of magnitude greater than the equivalent toxicity of the individual drugs alone. Drugs with similar modes of action can have additive or potentiated toxicity in a mixture setting, and thus become ecotoxicologically significant. For example, a mixture of two non-steroidal anti-inflammatory drugs (NSAIDs) caused toxicity in *Daphnia magna* at concentrations that showed little or no effect when each NSAID was tested individually (Cleuvers, 2003).

1.2 Effluent Pharmaceuticals Chosen

Non-steroidal anti-inflammatory drugs are some of the most commonly used medications and many are supplied as over-the-counter drugs. More than 30 billion doses of NSAIDs were consumed last year in the United States alone, and 50,801 documented ingestions were made by children aged 5 years or younger (Mowry *et al.*, 2013). These effluent pharmaceuticals do not readily degrade and are not completely metabolised by the body, being excreted slightly transformed or unchanged into WWTPs (Heberer, 2002). The ecological risk of anti-inflammatory residues in waters and sediments was deemed to be higher than several other pharmaceuticals found in effluent (Hernando *et al.*, 2006). NSAIDs are common contaminants found in effluent, raw sewage, and waterways across the globe (Ternes, 2001). It is estimated that the annual production worldwide is around several kilotons (Cleuvers, 2004). The three specific NSAIDs chosen in this study were ibuprofen, naproxen and diclofenac. All three have been deemed high risk pollutants and are the most prevalent NSAIDs found in surface and groundwaters (Hebrer *et al.*, 1998; Herando *et al.*, 2006).

NSAIDs inhibit prostaglandin biosynthesis by inhibiting cyclooxygenase enzymes (COX) (Vane, 1996). Known side-effects of NSAIDs can include irritation of stomach lining and toxic effects on the kidneys. These side-effects generally occur when the constituent enzyme COX 1 is inhibited (Vane, 1996). COX 2 is induced by pro-inflammatory stimuli, and can be inhibited by NSAIDs to relieve pain and inflammation. The COX 2/COX 1 activity ratio determines the NSAIDs range of activities and side-effects. Better potency against COX 2 and a better COX 2/COX 1 activity ratio will thus likely have fewer side-effects and be more effective (Vane, 1996).

Ibuprofen inhibits two fatty acyl substrates that COX 2 oxygenates: 2-arachidonoylglycerol through non-competitive inhibition, and arachiadonic acid through competitive inhibition (Prusakiewicz *et al.,* 2009). Ibuprofen has a high affinity for COX 2, and is a relatively weak, rapidly reversible COX inhibitor (Prusakiewicz *et al.,* 2009). Decreased hepatic COX activity and increased liver somatic index have been associated with exposure of Japanese Medaka (*Oryzias latipes*) to ibuprofen (Flippin *et al.,* 2007).

Ibuprofen is a very ubiquitous pollutant and has been found in Montreal municipal sewage treatment effluent at concentrations up to 1.19 µg/L and in Ontario sewage effluent up to 85 µg/L (Metcalfe *et al.,* 2003; Gagne *et al.,* 2006). Globally, ibuprofen has been commonly found in waste water treatment plant effluents, for example ibuprofen was found to exceed 1 µg/L in Germany, with surface waters at lower concentrations (Ternes, 1998). In river water, ibuprofen was found at lower concentrations, ranging between 0.01 and 0.5 µg/L (Stumpf *et al.,* 1996). In Canada, concentrations of ibuprofen up to 22 µg/L have been found in treated effluent (Burn *et al.,* 2006). Concentrations of ibuprofen that affect development of *Hydra* have been found to be lower than concentrations affecting survival, with a reported teratogenic index (TI) of > 3 (Johnson *et al.,* 1987).

Naproxen has a typical NSAID mechanism of action and the parent compound has been found in Montreal municipal sewage treatment effluent at concentrations up to 0.217 µg/L, and in Ontario sewage effluent up to 12.5 µg/L (Weigal *et al.,* 2004; Gagne *et al.,* 2006). Globally, naproxen is also commonly found in wastewater treatment plant effluents, with reported concentrations exceeding 1 µg/L in Germany, while surface waters contained lower concentrations (Ternes, 1998). Photoproducts of naproxen are often more toxic than the parent compound (Isidon *et al.,* 2005). These photoproducts can decrease the survival and growth of

rotifers and crustaceans (Isidon *et al.,* 2005). Photoproducts would be more environmentally relevant to test, however naproxen's photoproducts are not commonly available for testing. Concentrations of naproxen that affect development of *Hydra* have been found to be lower than those affecting survival (Johnson *et al.,* 1987; Isidon *et al.,* 2005).

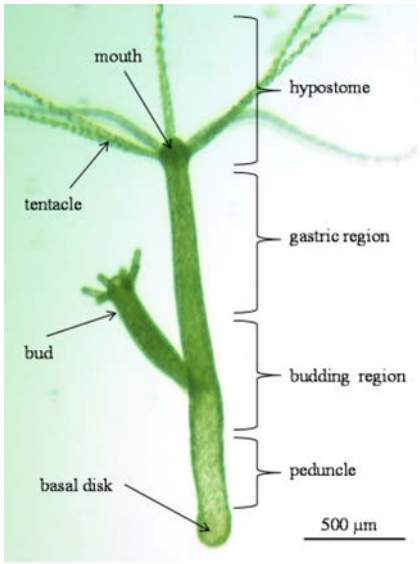
Diclofenac also has a typical NSAID mechanism of action and is commonly sold around the world. Approximately 75 tons of diclofenac is annually sold in Germany and in 2005 more than 4.4 kg of diclofenac per week was discharged into Berlin's surface waters through WWTPs (Ternes, 2001; Heberer & Feldmann, 2005). Diclofenac has been found in sewage effluent at concentrations exceeding 1.59 µg/L, and in river waters ranging from 0.01 to 0.5 µg/L (Stumpf *et al.,* 1996). In one German wastewater treatment plant effluent, diclofenac was found to exceed 1 µg/L, with lower concentrations reported in surface waters (Ternes, 1998). A major tributary of Greifensee Lake had concentrations of diclofenac up to 0.370 µg/L, while the outflow had up to 0.012 µg/L of diclofenac (Buser *et al.,* 1998). This effluent pharmaceutical has caused delays in hatching time in zebrafish (Hallare, 2004).

1.3 Test Organisms Chosen

Hydra are Cnidarian invertebrates from the Class Hydrozoa. The distinguishing feature of Cnidarian organisms are that they contain specialized cells that are used mainly for capturing prey. Cnidaria have 2 basic body forms: swimming medusa and sessile polyps, both of these forms are radially symmetrical and have mouths that are surrounded by tentacles. *Hydra* are considered to be sessile polyps and are only passively carried by water when food is scarce. *Hydra* are small, solitary, predatory animals, and are related to jellyfish and corals. Several species of *Hydra* have been found on all continents across the globe (Holstein *et al.,* 1990). Five different species have been described in Europe, while in North America *Hydra littoralis* and

Hydra carnea are most common and in Japan, *Hydra magnipapillata* have been discovered which closely resemble *Hydra vulgaris* of Europe (Holstein *et al.,* 1990).

The main body plan of a *Hydra* consists of two connected epithelial cell layers that are in contact with the aqueous environment (diploblastic). *Hydra* have an outer ectoderm, an acellular mesoglea layer, and an inner endoderm. The mouth (hypostome) is located at the top along with stinging cells (nematocysts) that are used to catch prey. A gastrovascular cavity is located in their gastric section that creates the *Hydra*'s hydrostatic skeleton and is the site for food digestion and nutrient absorption. They also have a budding region, peduncle section (between lowest bud and basal disc), a basal disc (foot like formation at bottom of the *Hydra*) and a simple nervous system that stretches throughout their body (see diagram of basic hydra structure below).



Hydra wait passively for small aquatic animals to brush against their tentacles; when contact is made the animal is immediately stung (Ewer, 1947). The *Hydra*'s mouth is opened by

a neuromuscular system in response to the association of glutathione with an external chemoreceptor, allowing the prey to be swallowed whole (Ewer, 1947; Bellis *et al.,* 1992). Hydra's population densities closely follow increases in their prey, zooplankton (Cuker & Mozley, 1981). *Hydra* have also been known to ingest cladocerans (water fleas), copepods (small crustaceans), rotifers (microscopic animals), larval fish and brine shrimp (Loomis & Lenhoff, 1956; Schwartz & Hebert, 1989; Link & Keen, 1995; Walsh, 1995; Elliot *et al.,* 1997). *Hydra* are normally found attached to sticks, rocks and plants and act as both predators and prey at the lower trophic levels (Elliot *et al.,* 1997). An example of a *Hydra* predator would be flatworms (Slobodkin & Bossert, 2001).

Two species of *Hydra* were chosen as test organisms to evaluate the toxicity of effluent pharmaceuticals due to *Hydra*'s ubiquitous global distribution. They are also found locally, in Lake Superior for example (Link & Keen, 1995). *Hydra* have also been used in several local and global studies that evaluated the toxicity of effluent pharmaceuticals. For example, the toxicity of industrial effluent found in the St. Lawrence River has been evaluated by researchers at Environmental Canada through the use of *Hydra* to evaluate toxicity (Trottier, 1995). Due to their wide distribution in freshwater systems, these invertebrates are environmentally relevant test organisms.

Hydra bioassays have been found to be more sensitive to specific toxicants than those performed with other invertebrates, vertebrates and plant organisms (Oberholster *et al.,* 2008). Despite *Hydra*'s sub-lethal endpoints being more sensitive than lethality, *Hydra* are not presently used in most formal monitoring programs (Arkhipchuk *et al.,* 2006). They are, however, commonly used in acute 96 hour toxicity tests. *Hydra* are easy to culture, with standardized culture methods published and they create genetically identical clones through asexual budding.

Asexual budding decreases statistical variation due to genetic differences and permits population reproduction effects of a potential toxicant to be determined in the laboratory (Holdway, 2005). *Hydra* have a simple diploblastic structure that allows xenobiotic substances to enter with ease. Through the use of a dissecting microscope, chronic effects of a xenobiotic can be evaluated using published morphological charts (Wilby, 1988).

Nevertheless, the use of *Hydra* does have limitations. *Hydra* are affected by several external parameters, for example temperature and light, so consistent monitoring and recording of abiotic factors throughout the experimental period is required. *Hydra* exhibit fast reproductive rates under favourable conditions by reproducing asexually (Holdway, 2005). Factors that contribute to optimal growth of *Hydra* include water conditions such as; temperature, pH, dissolved oxygen, the amount of prey provided and the ionic balance. Ideal conditions are: 6,000 ± µg/L dissolved oxygen, maximal water hardness of 750,000 µg $CaCO_3$/L, pH 6 to 8, temperature 20 to 30ºC and daily feeding (Loomis, 1953; 1954). There are only two ions required for growth; calcium and potassium (Lenhoff & Brown, 1970). Overall there are 4 criteria for good culturing: suitable culture condition, live food of high nutritional value, attachment of *Hydra* and asexual reproduction of *Hydra* (Lenhoff & Brown, 1970). Crowded *Hydra* have the tendency to detach more readily than sparsely populated *Hydra* cultures (Lenhoff & Brown, 1970). Each *Hydra* will normally eat 1 to 3 individual brine shrimp during a feeding period and the culture solution allows the invertebrate to carry out its functions properly (Campbell, 1961). For a healthy *Hydra* culture, the natural logarithm of the number of individuals plotted against time will have a linear trend (Stebbing & Pomroy, 1978).

During the asexual reproductive mode, the tissues of the *Hydra* are filled with stem cells that have a continuous renewal potential and create a bud. How many buds are produced can be

related to the size of the individual (Holdway, 2005). Sexual reproduction occurs under stressful environmental conditions through the production of both male and female gonads (Littlefield *et al.,* 1991). Sexual reproduction occurs in larger individuals more frequently and increases with a decrease in temperature (Littlefield *et al.,* 1991; Holdway, 2005). *Hydra* have the capacity to sense environmental conditions which allows for the switching between sexual and asexual reproduction (Schaible *et al.,* 2011).

The detoxification process of xenobiotics in *Hydra* is not well documented. It has been theorized that the diploblastic structure that allows ions to move easily throughout the *Hydra,* could permit diffusion to detoxify xenobiotics like metals (Walker *et al.,* 2006). Another theory is that the *Hydra* sequesters and then expels the xenobiotic through routinely discharging cells as new cells replace them, as shown with uranium (Hyne *et al.,* 1992). Low levels of heat shock proteins have been discovered in *Hydra* (Brennecke *et al.,* 1998). Expression of these proteins will increase when cells are exposed to elevated temperatures or stress. A lasting toxic effect from xenobiotic exposure only occurs when their capacity to counteract toxicity is exceeded (Stebbing & Pomroy 1978).

Techniques to identify individual species of *Hydra* have been developed through examining the structures of the nematocysts, morphology and body characteristics (Holstein *et al.,* 1990). Species of *Hydra* differ in several aspects including but not limited to; presence or absence of algal symbiosis, colour, size, ideal environmental conditions and food source. Species of *Hydra* that do not have an algal symbiosis are found in the zone of water that is located right above the sediment termed the benthic zone. This region is the lowest region found in a body of water in freshwater ecosystems and has the lowest oxygen content. The benthic zone contains the sediment surface along with some sub-surface layers. Organisms that live here are in close

relationship with the substrate bottom and many are permanently attached (Elliot *et al.*, 1997). Some examples of benthic organisms include crustaceans and some specific Cnidarian species like *Hydra littoralis* (brown *Hydra*).

Species of *Hydra* that possess an algal symbiot are generally found in the littoral zone, the highest zone of water that is closest to the shore. This region receives the most sunlight out of all the zones in a body of water and has the highest oxygen content. Organisms that live in this zone can be specifically adapted to capturing sunlight. Some examples include coral reefs and *Hydra viridissima* (green *Hydra*), both of which have algal symbiots that depend heavily on temperature or the presence of light (Lenhoff & Brown, 1970).

Green *Hydra* are considered to be mixotrophic, they are organisms that can use a mix of different sources of energy and carbon. Green *Hydra* contain an algal symbiot and it is because of the algal presence that they are green in colour (Whitney, 1907). The algal component in green *Hydra* provides maltose (carbon source) and oxygen (energy source) to its host. The advantages that the algal component provides are not necessary for sustaining growth and are used as a supplemental source. The algal component is lost if the green *Hydra* are kept in the dark, however, these *Hydra* can still survive and reproduce if placed in appropriate environmental conditions (access to prey, access to ions required etc.) (Whitney, 1907; Lenhoff & Brown, 1970). If green *Hydra* are starved they will likely live longer than *Hydra* that don't possess symbiots, however, with indefinite periods without access to prey, they will eventually perish (Lenhoff & Brown, 1970).

Hydra that do not have algal symbiots (brown *Hydra* for example) are considered to be heterotrophic, they are organisms that cannot fix carbon and uses organic carbon for growth.

They would be considered chemoheterotrophs since they do not use light for energy but use inorganic/organic energy sources.

Hydra viridissima (green *Hydra*) and *Hydra littoralis* (brown *Hydra*) were selected for use in this study (Ward's Natural Science Ltd., St. Catharines, Ontario, Canada). Green *Hydra* have symbiotic *Chlorella* algae that provide the *Hydra* with photosynthetic abilities, with the algal component mostly located in the upper body column (Lenhoff & Brown, 1970; Habetha *et al.*, 2003). Due to this symbiosis, green *Hydra* require oxygen for carbohydrate metabolism, which they utilize as their main food source. The maltose supplied by the algae provides green *Hydra* with an advantage during periods of starvation and permits them the ability to live in clear, slow moving water (Holdway *et al,* 2001; Habetha *et al.*, 2003). Green *Hydra* are smaller in size than brown *Hydra* and prefer temperatures around 30ºC. Brown *Hydra* do not possess algal symbiots, are larger in size, and are generally healthier (elongated tentacles, able to ingest food etc.) than green *Hydra* during winter months. They require flowing water to maintain their asexual reproduction and utilize lipids as their main food source. In a laboratory culture both green and brown *Hydra* are fed live brine shrimp which contains the nutrients needed for both species of *Hydra* to be successfully cultured (Lenhoff & Brown, 1970).

1.4 Endpoints Evaluated

Morphology scores are commonly used to examine acute toxicity in *Hydra* while reproduction, feeding, and biochemical endpoints are generally used to examine non-lethal and chronic effects (Quinn *et al.,* 2009). Morphology, prey ingestion, reproduction and attachment were the four endpoints used in this study.

A morphological scale is used to categorize how a substance impacts morphology, using a binocular microscope to quantify any observed morphological changes over time (Wilby 1988; Blaise & Kusui, 1997). This scale contains 5 ranges: the first range of scores (8 to 10) is 'normal' (elongated tentacles and body); the second range is considered to have bulbed or clubbed tentacles (scores 6 to 8); third range has shorted tentacles (score 6); forth range is called the tulip phase (score 5), and the fifth range is disintegration (score 0). Scores 6 to 10 are considered reversible, sub-lethal indicators, while scores of 0 to 5 are considered irreversible, lethal indicators (Blaise & Kusui, 1997). The tulip stage is when the animal does not recover and will disintegrate even when rescued from the xenobiotic and placed in normal medium (Johnson & Gabel, 1983). Thus the clubbed tentacle stage is the mark of sub-lethality while the tulip stage is the mark of lethality (Trottier *et al.,* 1997).

Hydra reproduction, population growth rate, abundance and composition are positively correlated with prey ingestion (Juchelka & Snell, 1994). Higher rates of feeding will result in logarithmic rates of growth (Loomis, 1954). *Hydra* use their nematocysts that inject toxins to capture prey, then the prey are transported to the mouth, ingestion occurs in the gastrovascular cavity, followed by the ejection of the undigested exoskeleton (Lenhoff, 1961). This process is initiated with the association of glutathione (GSH) with an external chemoreceptor (Bellis *et al.,* 1992). Oxidative stress or a xenobiotic conjugating with the GSH will hinder the feeding process (Pascoe *et al.,* 2003; Quinn *et al.,* 2004; Quinn *et al.,* 2008a;Quinn *et al.,* 2007; Quinn *et al.,* 2009).

Reproduction is determined by observing the changes in the number of *Hydra* over time and shows the biological effects the substance has on the animal (Stebbing & Pomroy, 1978;

Holdway 2005). The concentration at which a substance has a statistically significant effect can be evaluated using the hydra population reproduction toxicity test method (Holdway, 2005).

Attachment is not a common endpoint used, but *Hydra* require attachment in order to feed, grow and reproduce. *Hydra* have an adhesive gland, that attaches onto the surface of objects, in their basal disc (Muller, 1996). A decrease in attachment has been shown to occur in the presence of xenobiotics (Quinn *et al.,* 2007).

1.5 Comparable Studies

Quinn *et al.* (2009) investigated the effects of a mixture of 11 pharmaceuticals on *Hydra* over 96 hours at concentrations of 1 to 10,000 times the concentrations found in Montreal municipal sewage effluent using a 0.62% ethanol carrier (carrier that was toxic to *Hydra*). An increase in morphological changes occurred with an increase in concentration (up to 1,000 times). Feeding and *Hydra* number decreased at 10,000 times only, with attachment showing decrease at ≥ 1,000 times. The 96 hour EC_{50} equivalence (concentration affecting 50% of the population) was 506 µg/L and 92 µg/L for ibuprofen and naproxen, respectively.

Quinn *et al*. (2008a) investigated the effects of a mixture of 11 pharmaceuticals on budding *Hydra* over 96 hours at concentrations between 100 µg/L and 50,000 / 100,000 µg/L using DMSO or ethanol as the solvent. Ibuprofen was found to be more toxic than naproxen. Morphological changes and prey ingestion increased with an increase in exposure. Attachment decreased at 1,000, 10,000, and 25,000 µg/L and *Hydra* number decreased at 5,000, 10,000 and 100,000 µg/L of ibuprofen.

Quinn *et al.* (2008b) used several pharmaceuticals that ranged between 100 and 50,000 / 100,000 µg/L and used 3 different solvents (DMSO, ethanol and acetone) to specifically examine

Hydra regeneration. Morphological scores decreased with increase in exposure concentration. The half maximal inhibitory concentrations (the effectiveness of a substance in inhibiting a specific biological function, IC_{50}) were 3,840 µg/L and 4,900 µg/L for ibuprofen and naproxen, respectively, which is similar to the adult EC_{50} reported in Quinn *et al* (2008a).

Pascoe *et al.* (2003) exposed *Hydra* for 7 days to 10 pharmaceuticals in *Hydra* medium with ethanol (0.01-10%) as the carrier and used cadmium as a reference toxicant. Morphology and feeding decreased with an increase in concentration. No effects on the number of buds produced were observed in the different drug treatments.

Karntanut & Pascoe (2000) used two different methods in order to determine the LC_{50} (concentration that is lethal to 50% of the population) and EC_{50} of copper. The first was using a conventional determination (mortality) and the second was based on a scoring procedure of progressive changes in structure (morphology). The LC_{50} and EC_{50} determined for copper was 32 µg/L and 19 µg/L, respectively.

Section 2: Research Questions

More than 30 billion doses of NSAIDs were flushed down the toilet last year in the United States (Mowry *et al.,* 2013). Is this impacting non-target species? Two of the three selected NSAIDs have EC_{50} values that are classified as "very toxic to aquatic organisms" based on the European Union Directive 93/67/EEC (CEC, 1996). There are several research gaps in the literature including how these selected NSAIDs affect *Hydra* at the population level. These experiments would also show if NSAIDs are toxic to *Hydra* at environmentally relevant concentrations as well as any differences in toxicity between species.

The short term goals of this research are to determine if 7 days of exposure to 3 different NSAIDs are toxic to *Hydra*, by evaluating attachment, morphology, prey ingestion and reproduction. These experiments will also determine if the species of *Hydra* impacts the toxicity of these NSAIDs. Specific species of *Hydra* possess advantageous abilities such as algal symbiots that could impact a xenobiotics' toxicity. The results found could help in providing a rationale for the development of processes to eliminate pharmaceuticals by identifying if NSAIDs are a high risk pollutant to invertebrates populations.

A long term goal of this research would be the creation of a bioassay utilizing *Hydra* to test sewage effluents or wastewater for these types of pharmaceuticals. *Hydra* are both predator and prey in the food web. If negative effects of NSAIDs are shown at this level of the food web, population densities of both their prey and their predators could be negatively impacted as well.

There were two main research questions that were addressed in this study:

1. Do select NSAIDs cause chronic (exposure period exceeding 96 hours) toxicity in *Hydra*? The null hypothesis is that NSAIDs are not toxic to aquatic organisms.

2. Is there a difference in NSAID toxicity between different species of *Hydra*? The null hypothesis is that there is no difference in NSAID toxicity between green and brown *Hydra.*

The major experimental objectives of this study were:

a. To ensure stock sensitivity did not vary greatly over time and that *Hydra* behaved in a predictable and previously documented fashion to a known reference toxicant, and
b. To observe the chosen endpoints (morphology, attachment, prey ingestion and reproduction) during a chronic exposure to various concentrations of ibuprofen, naproxen and diclofenac using two species of *Hydra*.

Section 3: Methodology

3.1 Culturing Hydra

Hydra were cultured on a laboratory bench and in a temperature control room in the aquatic toxicology lab at UOIT. The *Hydra* were reared in lab water that had undergone water treatment including charcoal filtration, water softening and reverse osmosis filtration, followed by the addition of magnesium and calcium to bring the pH to 7.5.

In order to culture the *Hydra*, covered glass dishes were used to minimize contamination and evaporation. Since abiotic modifying factors can influence *Hydra* populations, environmental conditions were optimized so that growth rate is affected mainly by food availability (Lenhoff & Brown, 1970). This was done by systematically adjusting the light settings and cleaning processes until optimal population growth conditions were achieved. Normal background growth rates under defined laboratory conditions were observed by utilizing 10 budding *Hydra* of each species held in covered glass bowls. Animals were fed daily and rinsed carefully so that there was no loss of *Hydra* or buds. The number of *Hydra* was recorded daily over a 5 day period. Based on Lenhoff & Brown (1970) the logarithmic growth rate was calculated as:

$$k \text{ (logarithmic growth rate constant)} = 0.693 / T \text{ (doubling time)}$$

3.2 Reference Toxicant Test

The purpose of this test was to ensure stock sensitivity did not vary greatly over time and that *Hydra* behaved in a predictable and previously documented fashion to a known reference toxicant. Periodically, a reference toxicant test was performed for both species of *Hydra* throughout the experimental period. Tests performed at the beginning of the experimental period

(2012) used 10 mL of copper sulphate (0.0026 to 0.26 µg/L) in triplicate using petri dishes with 10 *Hydra* per species for each nominal concentration for a period of 5 days. EC_{50} and LC_{50} values were determined by concentration response curves based on the morphology scores and the number of *Hydra* that were recorded daily. Tests performed in the middle of the experimental period (2013) were performed with 4 mL of copper sulphate (0 to 1,000 µg/L) in triplicate using 12 well plates with 10 *Hydra* per species for each nominal concentration for a period of 7 days. EC_{50} and LC_{50} values were also determined by concentration response curves based on the morphology scores and the number of *Hydra* that were recorded daily.

3.3 7 Day Exposure Toxicity Test

The purpose of this test was to observe endpoints chosen (morphology, attachment, and prey ingestion) during a chronic exposure to ibuprofen, naproxen and diclofenac with two species of *Hydra.* Randomness and experimenter blinding were incorporated in all phases of every test to ensure experiments were free of bias (Holdway, 2005). Approximately, 250 healthy non-budding *Hydra* were set aside the day before the experiment and were not fed 24 hours prior to the test nor during the duration of the test. Two sets of experiments (1 set of triplicate concentrations each) were run for each species at the same time, giving an n of 6. Test solution concentrations that ranged from 0 µg/L to 9,000 / 10,000 µg/L were created using lab water with a positive control (copper) and a negative control (lab water) for each experiment run. A blinded, randomizing procedure was utilized to ensure that the experiment was free of bias. An aliquot of 4 mL of each blinded concentration (random letter given) was placed into each of the 6 wells assigned to that letter that were randomly positioned among the multi-well plates. An aliquot of 10 mL of each blinded concentration was placed in corresponding letter labelled petri dishes

(transfer wells). The use of transfer wells minimized the occurrence of unwanted treatment dilution.

Hydra were drawn using a Pasteur pipette and 15 animals were distributed at a time into each lettered transfer well. From the transfer wells of each blinded concentration, 5 individual *Hydra* were selected and placed into the corresponding letter labelled wells on the multi-well plates. A sample size of 5 *Hydra* per well was chosen based on the sample size commonly used in comparative studies. Power analysis also showed that this sample size was appropriate. Then the multi-well plates were covered to prevent evaporation. Observations like number attached and morphology scores were recorded for 7 days. After the 7 day exposure, one *Hydra* from every well was placed into a new 12 multi-well plate that had 4 mL of lab water and 5 individual live brine shrimp inside. The number of brine shrimp left in each well was recorded at half hour intervals for 2 hours. This protocol was repeated for each drug and each species twice, giving an overall n of 12 and a total of 4 experiments run.

Ability to pool experiments together was determined by a two-way analysis of variance (ANOVA). If pooling data was acceptable, then a one-way ANOVA and *Post Hoc* tests were run in order to determine if there were any statistically significant differences between treatment groups. Regression or Spearman rank correlation was used in order to determine relationships between the variables. If not acceptable to pool, then a one-way ANOVA and *Post Hoc* tests were run in order to determine if there were any statistically significant differences between treatment groups. Regression or Spearman Rank Correlation was used for each experiment individually in order to determine relationships between the variables. Morphology was the endpoint where non-parametric tests had to be used regardless of normally, due to the ordinal nature of the data. Morphology is scored data and thus only non-parametric tests were

acceptable. The alpha value used for all tests was 0.05 and the average of each experiment (or pooled average) and standard error were displayed graphically.

3.4 Population Reproduction Test

The purpose of this test was to observe reproduction during a chronic exposure to ibuprofen, naproxen and diclofenac with two species of *Hydra.* Randomness and experimenter blinding were incorporated in all phases of every test to ensure experiments were free of bias (Holdway, 2005).

Population reproduction tests were performed with 10 mL of the selected NSAID (0 µg/L to 9,000 / 10,000 µg/L) in triplicate using 6 well plates with 5 budding *Hydra* per species for each nominal concentration for a period of 7 days. 100 % renewal of each concentration was made daily after observations were recorded and 100 to 200 µL of live nauplii brine shrimp was provided after each daily renewal. Rates of reproduction, lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC) were calculated based on the number of *Hydra* that were recorded daily.

Rate of reproduction (k) was calculated by using the equation:

$$k = [\log_e(ny) - \log_e(nx)] / [ty - tx]$$

where nx = number of hydra on first day and tx & ny = the number after y-x days (ty).

LOEC and NOEC values help to describe the toxicity that a xenobiotic has on the test subject. LOEC and NOEC values have limitations in their application to risk assessment and are susceptible to experimental design. The NOEC is the highest concentration to which there is no statistically significant effect on the animal. The LOEC is the lowest concentration to which

there is a statistically significant effect on the animal. The LOEC and NOEC values were determined based on the reproduction of *Hydra*. To calculate the LOEC, the rate of reproduction (k, equation above) of each *Hydra* in each well is calculated and the average rate of reproduction (mean k) is found for each concentration.

The difference (d) from the control is then calculated by subtracting each mean k value by the mean k value found for the negative control. The d values collected for each concentration are then divided by the standard error (SE) calculated for each concentration tested (d/SE ratios). Next, a comparison of the d/SE ratios to critical values occurs by ordering the d/SE ratios (from smallest to largest) against a set of critical values (1.79, 1.96, 2.14 …). Any ordered d/SE ratio that exceeds their compared critical value is statistically significant. The LOEC is the lowest concentration that is statistically different from controls ($p \leq 0.05$) and the NOEC is the next lowest test concentration.

3.5 Species Comparison

Experiments with both species of *Hydra* were run, with one exception, at the same time and under the same environmental conditions, allowing for direct comparison of toxicity. The exception was in the population reproduction tests for ibuprofen, the tests for each species were run in different environmental conditions. The green *Hydra* population reproduction tests were run in a temperature control room while the brown *Hydra* population reproduction tests were run on a main laboratory bench.

Section 4: Results

4.1 Culturing Hydra

The average logarithmic growth rates for green and brown *Hydra* stock cultures were 0.3 and 0.1, respectively. The average doubling times for green and brown Hydra were 2.3 and 6.4 days, respectively. Good quality control assessment requires that the *Hydra's* reproduction rate is comparable to natural freshwater reproduction rates of approximately 0.3 to 0.4 (Trottier *et al.,* 1997). Green *Hydra* produced at higher rates and reached the target rate while brown *Hydra* did not.

4.2 Reference Toxicant Test

The average LC_{50} values for green and brown *Hydra* were 12.6 µg/L and 14.0 µg/L, respectively (Figure 1). The average EC_{50} values for green and brown Hydra were 10.4 µg/L and 21.8 µg/L, respectively (Figure 1). There was a 4.2 µg/L and 1 µg/L difference in green and brown *Hydra*'s lethal sensitivity over time, respectively. There was a 10.7 µg/L and 24.5 µg/L difference in green and brown *Hydra*'s sub-lethal sensitivity over time, respectively. A statistically significant change over time occurred in green *Hydra*'s sub-lethality endpoint (morphology) ($p = 0.03$).

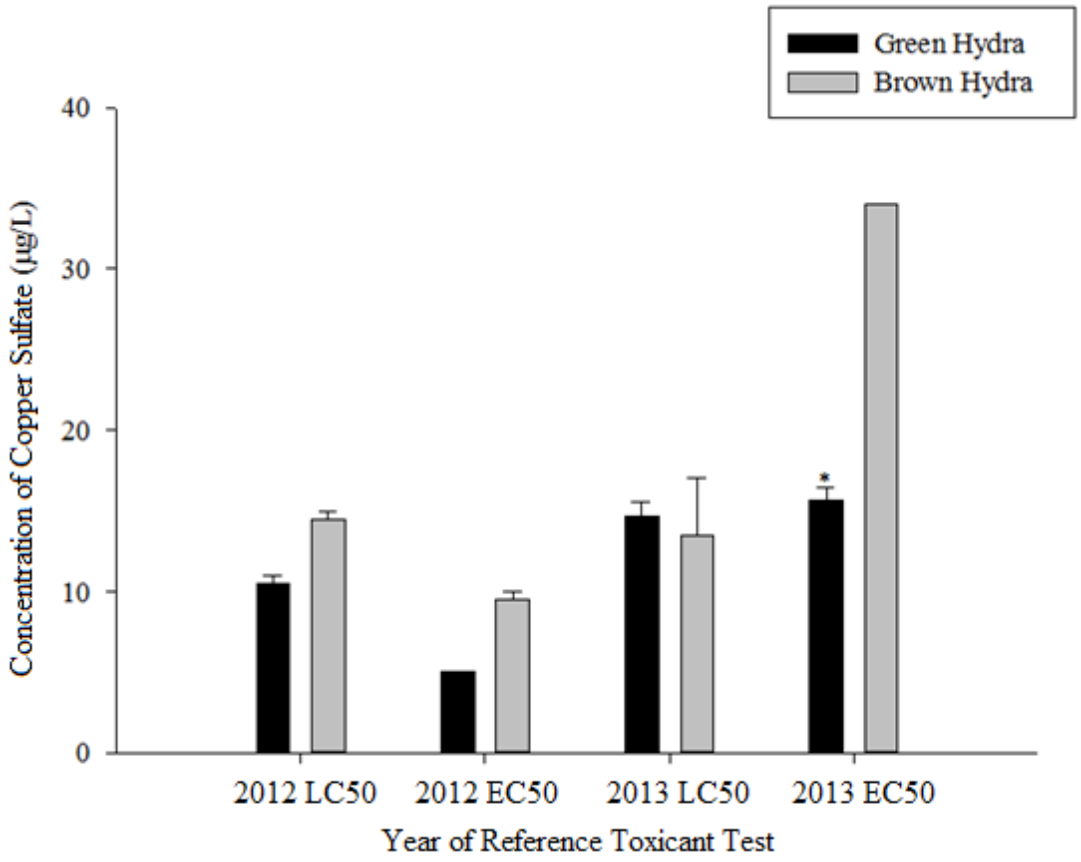


Figure 1: Reference toxicant tests for both green and brown *Hydra* over time during 5 or 7 day exposures to copper sulphate. The EC_{50} values for green *Hydra* showed a statistically significant change over time (*p = 0.03). The solid bars reprsent the average value found for 3 petri dishes/wells (total of 30 *Hydra*) per concentration and the error bars represent standard error.

4.3 7 Day Exposure Toxicity Test

Positive Control

An EC_{50} concentration of copper (10 µg/L) was used as a positive control in all 7 day exposure toxicity tests and was determined from the reference toxicant test results. The ibuprofen 7 day exposure toxicity tests were the first set of experiments to use copper as a positive control. Due to the lack of predicted response in the ibuprofen tests, the concentration of copper used was increased. This increase in concentration allowed for the expected response associated with copper, a decrease in morphological scores, as seen in the naproxen and diclofenac 7 day exposure toxicity tests (Appendix A). Attachment and feeding behavior of green and brown *Hydra* showed a decline when exposed to copper (Appendix A).

Ibuprofen

Attachment, morphology and prey ingested by green *Hydra* were evaluated in the four experiments run after 7 days of exposure to various concentrations of ibuprofen (Appendix B: i). Attached green *Hydra* in experiments 1 to 4 were normally distributed and based on the results from a 2-way ANOVA, the experiments were analyzed separately (Figure 2). Average number of attached green *Hydra* in the third experiment, using a 1-way ANOVA, was not the same in all concentrations tested ($p = 0.001$, $F = 9.2$) (Figure 2). Attachment of green *Hydra* in the third experiment was significantly higher at 10,000 µg/L compared to all other concentrations tested, determined through *Post Hoc* analysis ($p = 0.01$). No relationship was found between attachment of green *Hydra* and ibuprofen when regression was used.

Morphology scores of green *Hydra* in experiments 1 to 4 were analyzed separately with non-parametric tests (Figure 3). None of the experiments showed any statistically signficant results.

Average number of prey ingested by green *Hydra* 2 hours after 7 days of exposure to various conentrations of ibuprofen in experiments 1 to 4 were normally distributed and based on the results from a 2-way ANOVA, the experiments were pooled and analyzed together (Figure 4). Average number of prey ingested by green *Hydra*, using a 1-way ANOVA, was the same for all concentrations tested ($p = 0.13$, $F = 1.76$). No relationship was found between prey ingested by green *Hydra* and ibuprofen when regression was used.

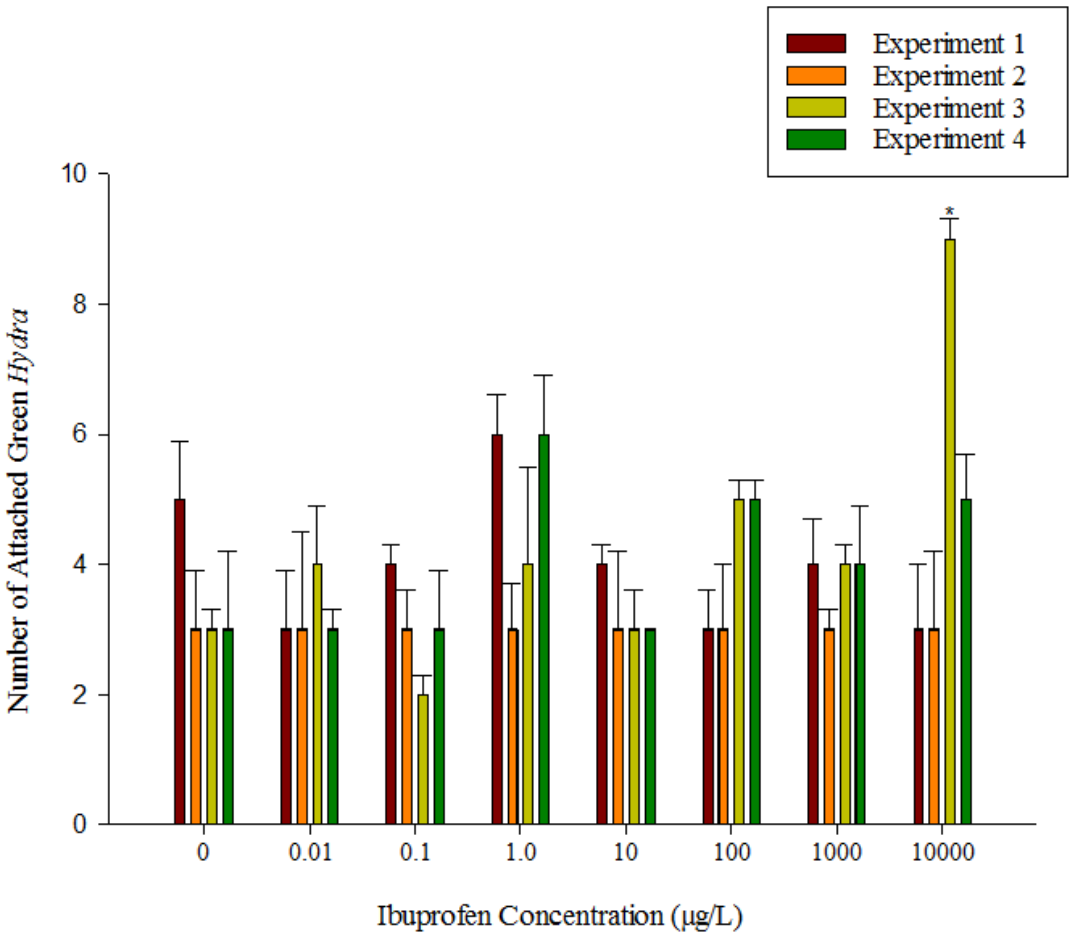


Figure 2: **Average number of attached green *Hydra* in experiments 1 to 4 after 7 days of exposure to various concentrations of ibuprofen.** Attachment of green *Hydra* was significantly higher at 10,000 µg/L in the third experiment compared to all other concentrations tested determined through *Post Hoc* analysis (*p = 0.01). The solid bars represent the average value found for 3 wells (total of 15 *Hydra*) per concentration and the error bars represent standard error. A positive control (copper) was run during each experiment to ensure that *Hydra* behaved in a predicted and previously documented way.

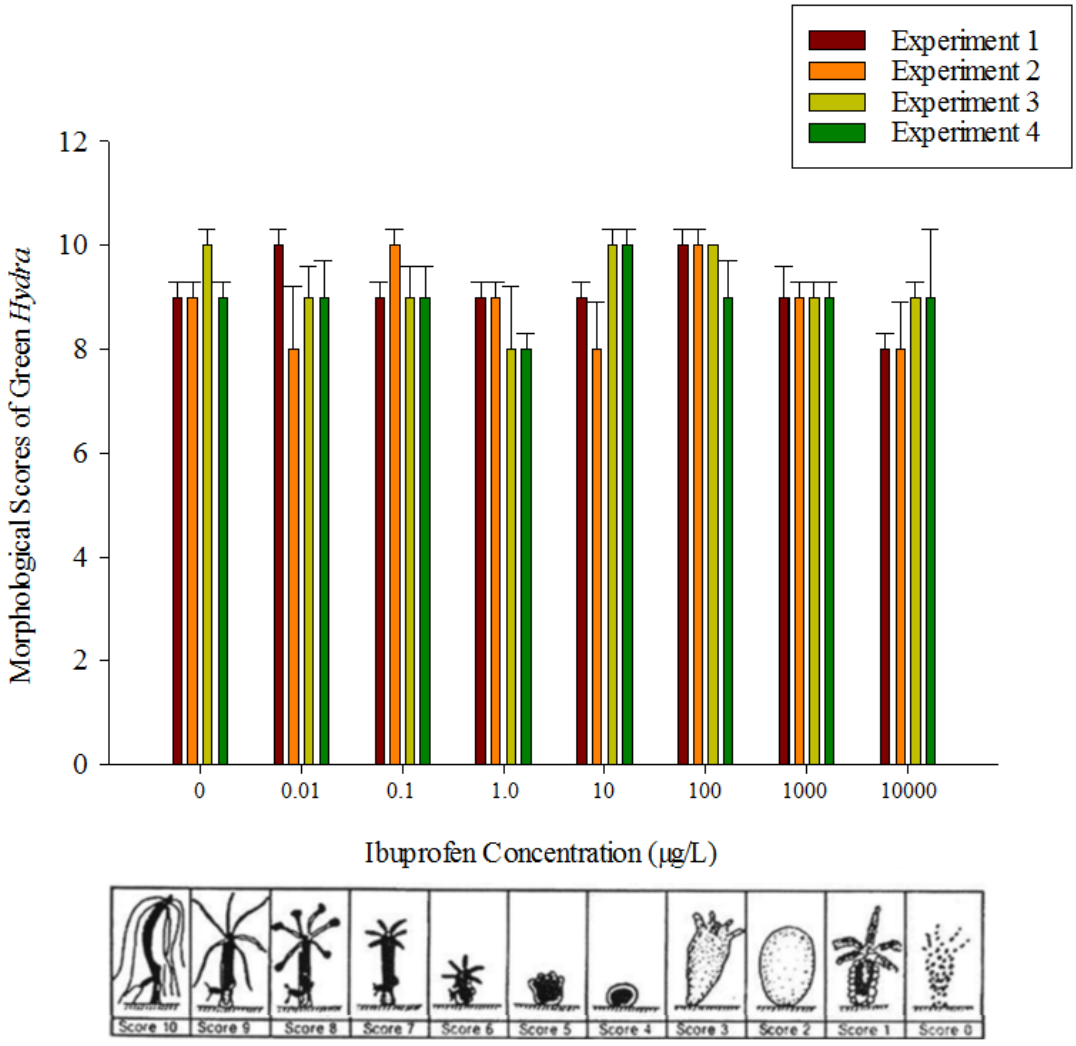


Figure 3: **Average morphology scores of green *Hydra* in experiments 1 to 4 after 7 days of exposure to various concentrations of ibuprofen.** No statistically significant results were found. The solid bars represent the average value found for 3 wells (total of 15 *Hydra*) per concentration and the error bars represent standard error. A positive control (copper) was run during each experiment to ensure that *Hydra* behaved in a predicted and previously documented way.

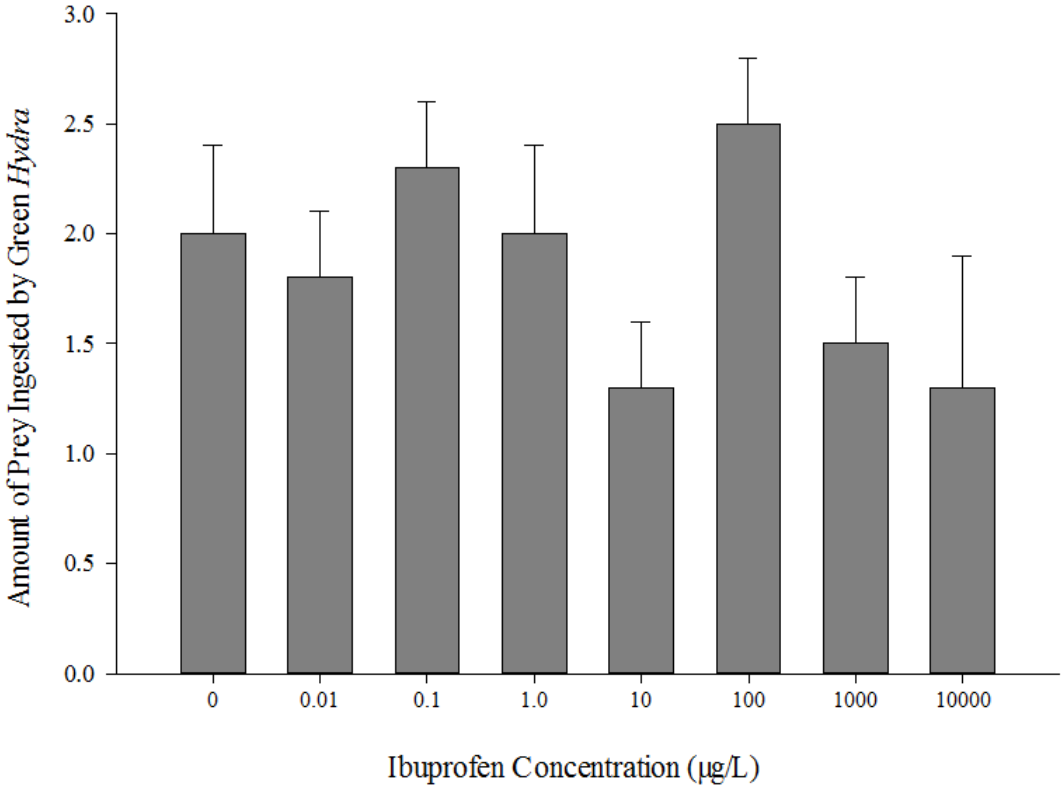


Figure 4: **Average number of prey ingested by green *Hydra* 2 hours after 7 days of exposure to various concentrations of ibuprofen in experiments 1 to 4 were pooled.** Prey ingested by green *Hydra* was comparable in all concentrations tested ($p = 0.13$). The solid bars represent the average value found for 12 wells (total of 60 *Hydra*) per concentration and the error bars represent standard error. A positive control (copper) was run during each experiment to ensure that *Hydra* behaved in a predicted and previously documented way.

Attachment, morphology and prey ingested by brown *Hydra* were evaluated in the four experiments run after 7 days of exposure to various concentrations of ibuprofen (Appendix B: ii). Attached brown *Hydra* in experiments 1 to 4 were normally distributed and based on the results from a 2-way ANOVA, the experiments were analyzed separately (Figure 5). Average number of attached brown *Hydra* in the second experiment, using a 1-way ANOVA, was not the same in all concentrations tested ($p = 0.04$, $F = 3.3$). A negative correlation was found between attached brown *Hydra* and ibuprofen in the second experiment, using regression ($b^* = -0.41$, $p = 0.04$). Average number of attached brown *Hydra* in the fourth experiment, using Spearman Rank Correlation, showed a positive correlation with ibuprofen (Spearman's rho = 0.43, $p = 0.04$).

Morphology scores of brown *Hydra* in experiments 1 to 4 were analyzed separately with non-parametric tests (Figure 6). Average morphology scores of brown *Hydra* in the second experiment, using Spearman Rank Correlation, showed a negative correlation with ibuprofen (Spearman's rho = -0.42, $p = 0.04$).

Average number of prey ingested by brown *Hydra* 2 hours after 7 days of exposure to various concentrations of ibuprofen in experiments 1 to 4 were normally distributed and based on a 2-way ANOVA, the experiments were analyzed separately (Figure 7). Average number of prey ingested by brown *Hydra* in the second experiment, using Spearman Rank Correlation, showed a negative correlation with ibuprofen (Spearman's rho = -0.43, $p = 0.03$).

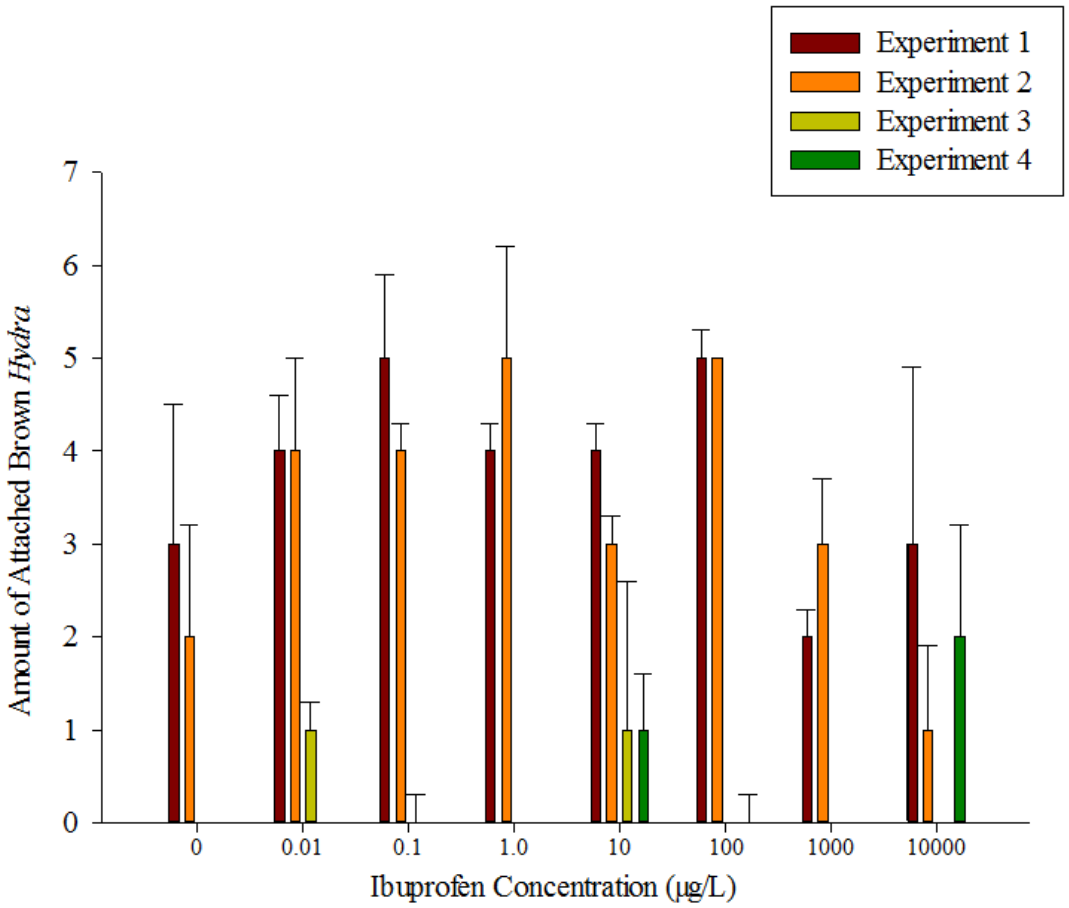


Figure 5: **Average number of attached brown *Hydra* in experiments 1 to 4 after 7 days of exposure to various concentrations of ibuprofen.** A negative correlation was found between attached brown *Hydra* and ibuprofen in the second experiment ($p = 0.04$). A positive correlation was found between attached brown *Hydra* and ibuprofen in the fourth experiment ($p = 0.04$). The solid bars represent the average value found for 3 wells (total of 15 *Hydra*) per concentration and the error bars represent standard error. A positive control (copper) was run during each experiment to ensure that *Hydra* behaved in a predicted and previously documented way. Any solid bars that are not shown represent zero *Hydra* attached for that experiment.

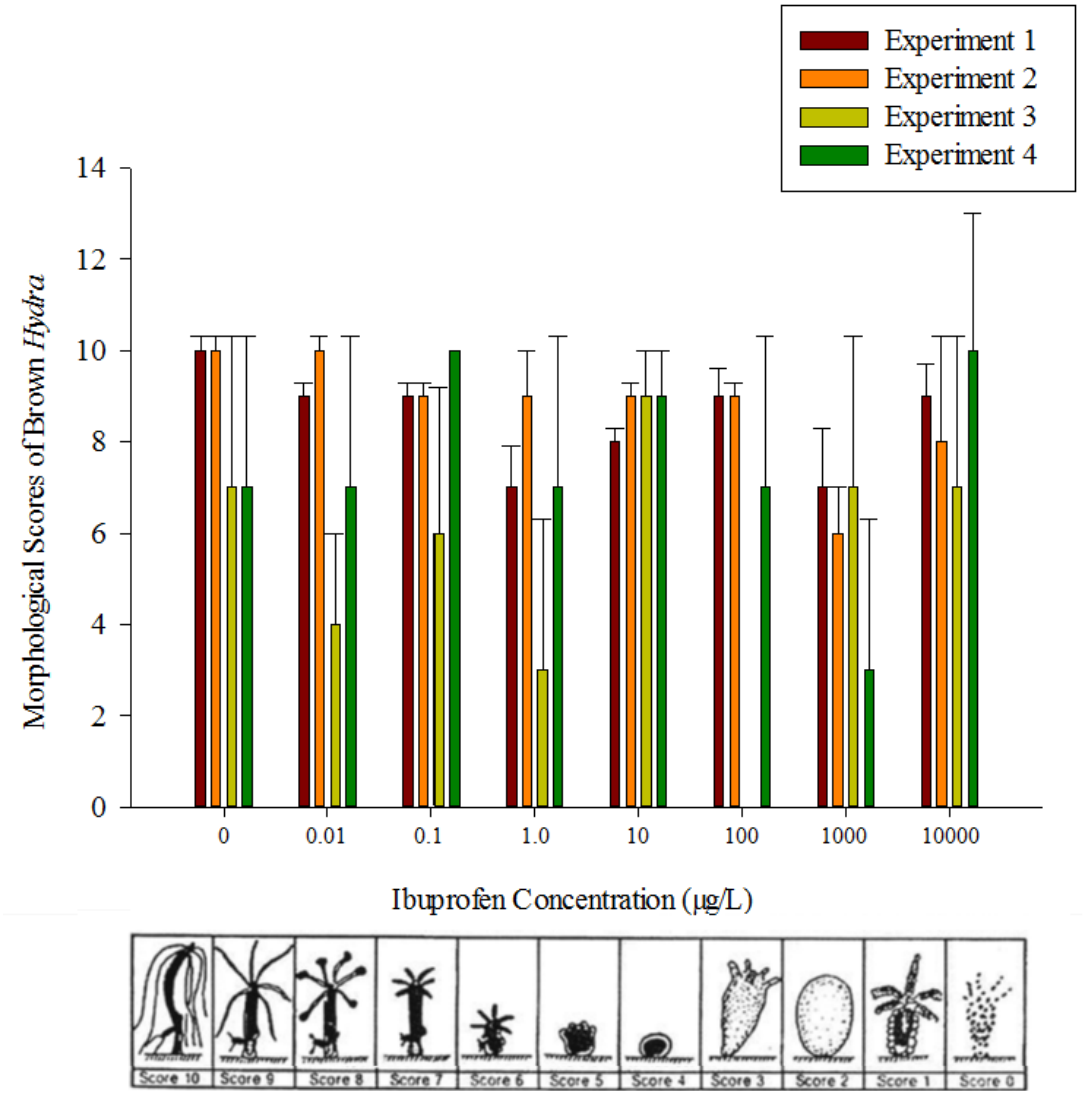


Figure 6: **Average morphology scores of brown *Hydra* in experiments 1 to 4 after 7 days of exposure to various concentrations of ibuprofen.** A negative correlation was found between morphology and ibuprofen in the second experiment ($p = 0.04$). The solid bars represent the average value found for 3 wells (total of 15 *Hydra*) per concentration and the error bars represent standard error. A positive control (copper) was run during each experiment to ensure that *Hydra* behaved in a predicted and previously documented way. Any solid bars that are not shown represent an average morphology score of zero for that experiment.

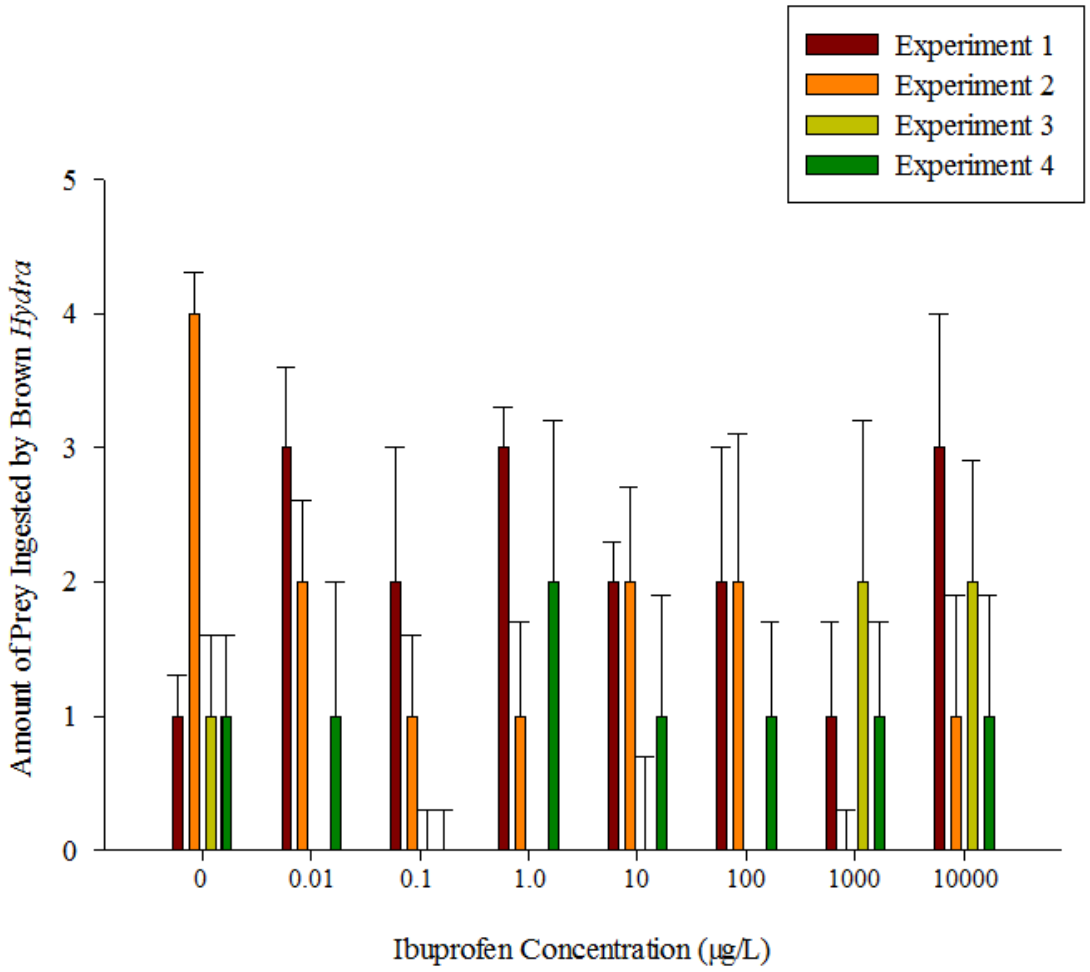


Figure 7: **Average number of prey ingested by brown *Hydra* 2 hours after 7 days of exposure to various concentrations of ibuprofen in experiments 1 to 4.** A negative correlation was found between prey ingested by brown *Hydra* and ibuprofen in the second experiment ($p = 0.03$). The solid bars represent the average value found for 3 wells (total of 15 *Hydra*) per concentration and the error bars represent standard error. A positive control (copper) was run during each experiment to ensure that *Hydra* behaved in a predicted and previously documented way. Any solid bars that are not shown represent the average amount of prey ingested is zero for that experiment.

Naproxen

Attachment, morphology and prey ingested by green *Hydra* were evaluated in the four experiments run after 7 days of exposure to various concentrations of naproxen (Appendix B: iii). Attached green *Hydra* in experiments 1 to 4 were normally distributed and based on the results from a 2-way ANOVA, the experiments were analyzed separately (Figure 8). None of the experiments showed any statistically signficant results.

Morphology scores of green *Hydra* in experiments 1 to 4 were analyzed seperately with non-parametric tests (Figure 9). Average morphology scores of green *Hydra* in the fourth experiment, using Spearman Rank Correlation, showed a negative correlation with naproxen (Spearman's rho = -0.43, $p = 0.04$).

Average number of prey ingested by green *Hydra* 2 hours after 7 days of exposure to various concentrations of naproxen in experiments 1 to 4 were normally distributed and based on a 2-way ANOVA, the experiments were analyzed separately (Figure 10). None of the experiments showed any statistically significant results.

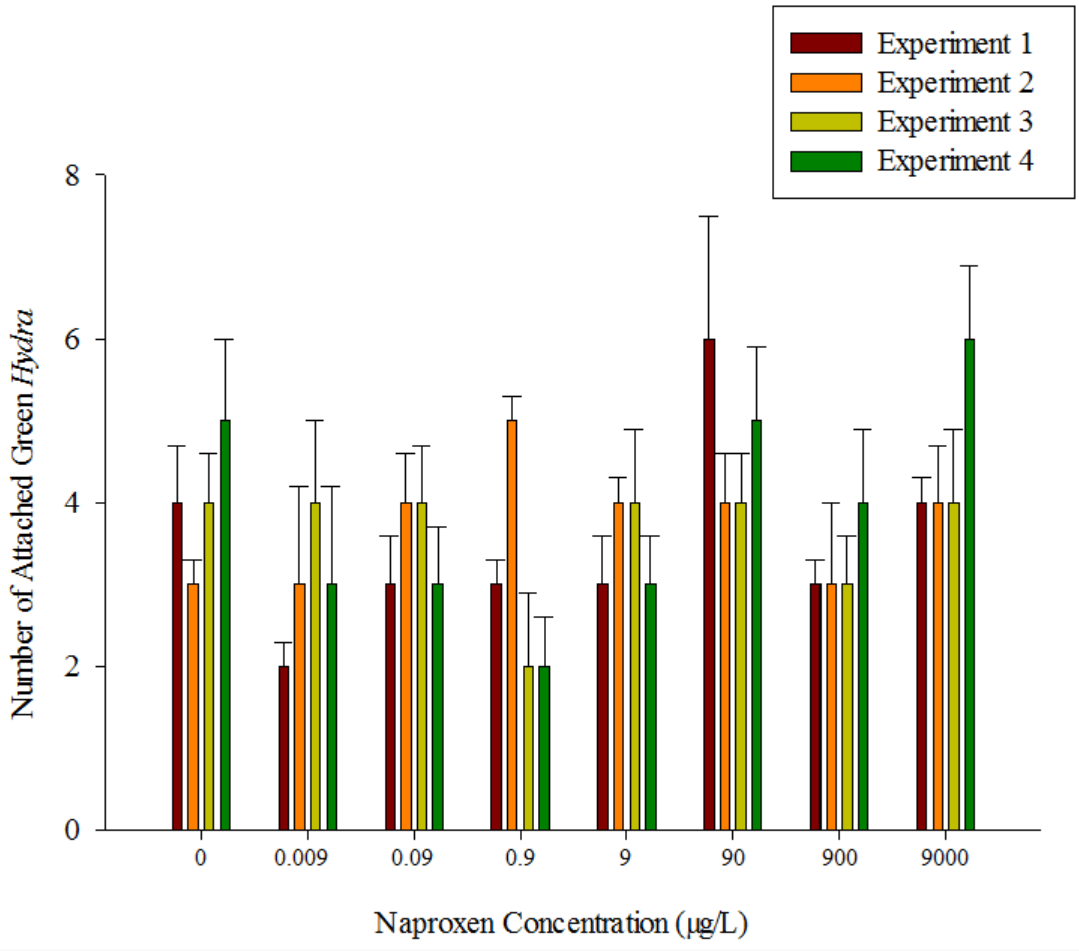


Figure 8: **Average number of attached green *Hydra* in experiments 1 to 4 after 7 days of exposure to various concentrations of naproxen.** No statistically significant results were found. The solid bars represent the average value found for 3 wells (total of 15 *Hydra*) per concentration and the error bars represent standard error. A positive control (copper) was run during each experiment to ensure that *Hydra* behaved in a predicted and previously documented way.

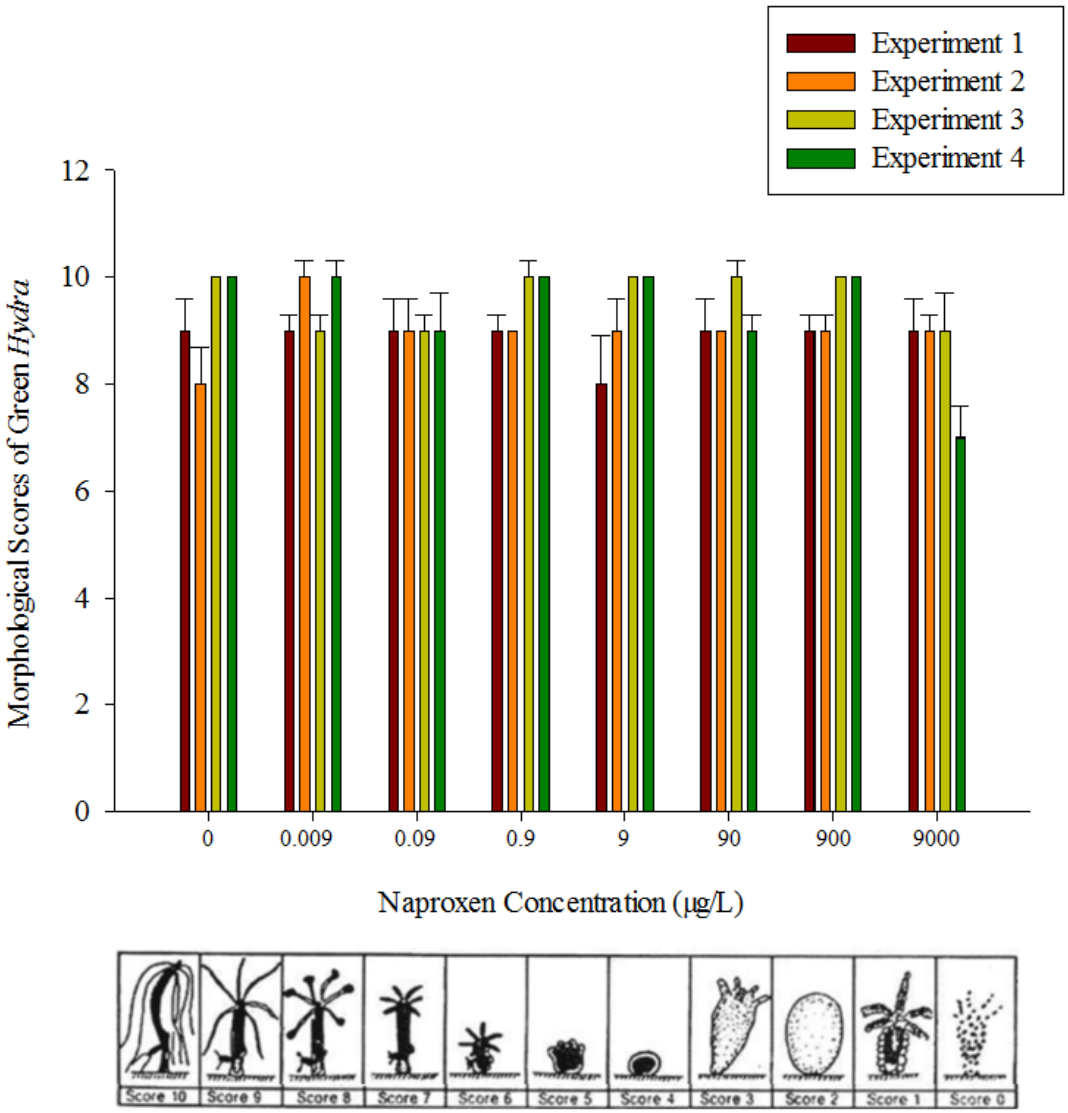


Figure 9: **Average morphology scores of green *Hydra* in experiments 1 to 4 after 7 days of exposure to various concentrations of naproxen.** A negative correlation was found between morphology scores of green *Hydra* and naproxen in the fourth experiment ($p = 0.04$). The solid bars represent the average value found for 3 wells (total of 15 *Hydra*) per concentration and the error bars represent standard error. A positive control (copper) was run during each experiment to ensure that *Hydra* behaved in a predicted and previously documented way.

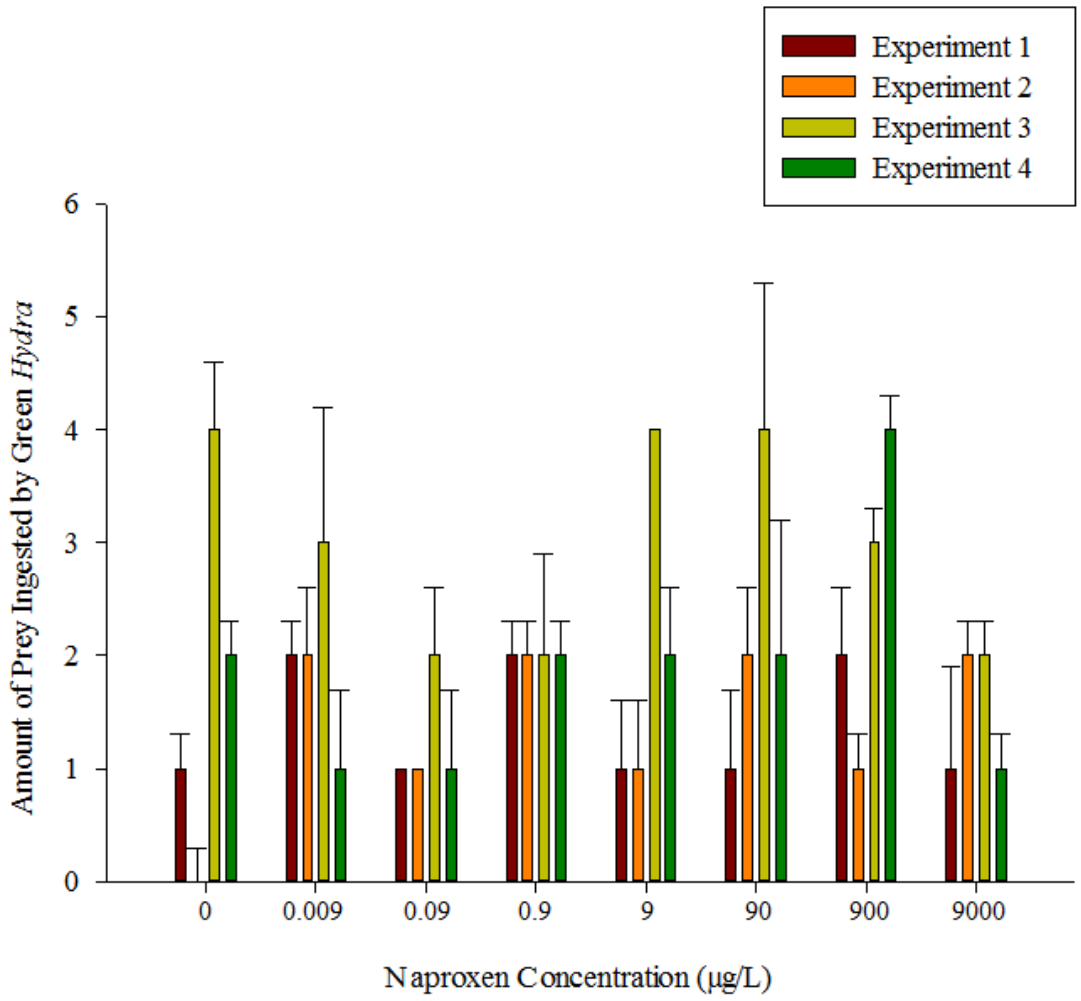


Figure 10: **Average number of prey ingested by green *Hydra* 2 hours after 7 days of exposure to various concentrations of naproxen in experiments 1 to 4.** No statistically significant results were found. The solid bars represent the average value found for 3 wells (total of 15 *Hydra*) per concentration and the error bars represent standard error. A positive control (copper) was run during each experiment to ensure that *Hydra* behaved in a predicted and previously documented way. Any solid bars that are not shown represent the average amount of prey ingested is zero for that experiment.

Attachment, morphology and prey ingested by brown *Hydra* were evaluated in the four experiments run after 7 days of exposure to various concentrations of naproxen (Appendix B: iv). Attached brown *Hydra* in experiments 1 to 4 were normally distributed and based on the results from a 2-way ANOVA, the experiments were analyzed separately (Figure 11). Average number of attached brown *Hydra* in the fourth experiment, using a 1-way ANOVA, was not the same in all naproxen concentrations tested ($p = 0.02$, $F = 4.79$). Attachment of brown *Hydra* was significantly higher at 900 µg/L compared to 90 µg/L of naproxen in the fourth experiment, determined through *Post Hoc* analysis ($p = 0.03$). No relationship was found between attachment of brown *Hydra* and naproxen when regression was used.

Morphology scores of brown *Hydra* in experiments 1 to 4 were pooled and analyzed together using non-parametric tests (Figure 12). None of the experiments showed any statistically significant results.

Average amount of prey ingested by brown *Hydra* 2 hours after 7 days of exposure to various concentration of naproxen in experiments 1 to 4 were normally distributed and based on a 2-way ANOVA, the experiments were analyzed separately (Figure 13). None of the experiments showed any statistically significant results.

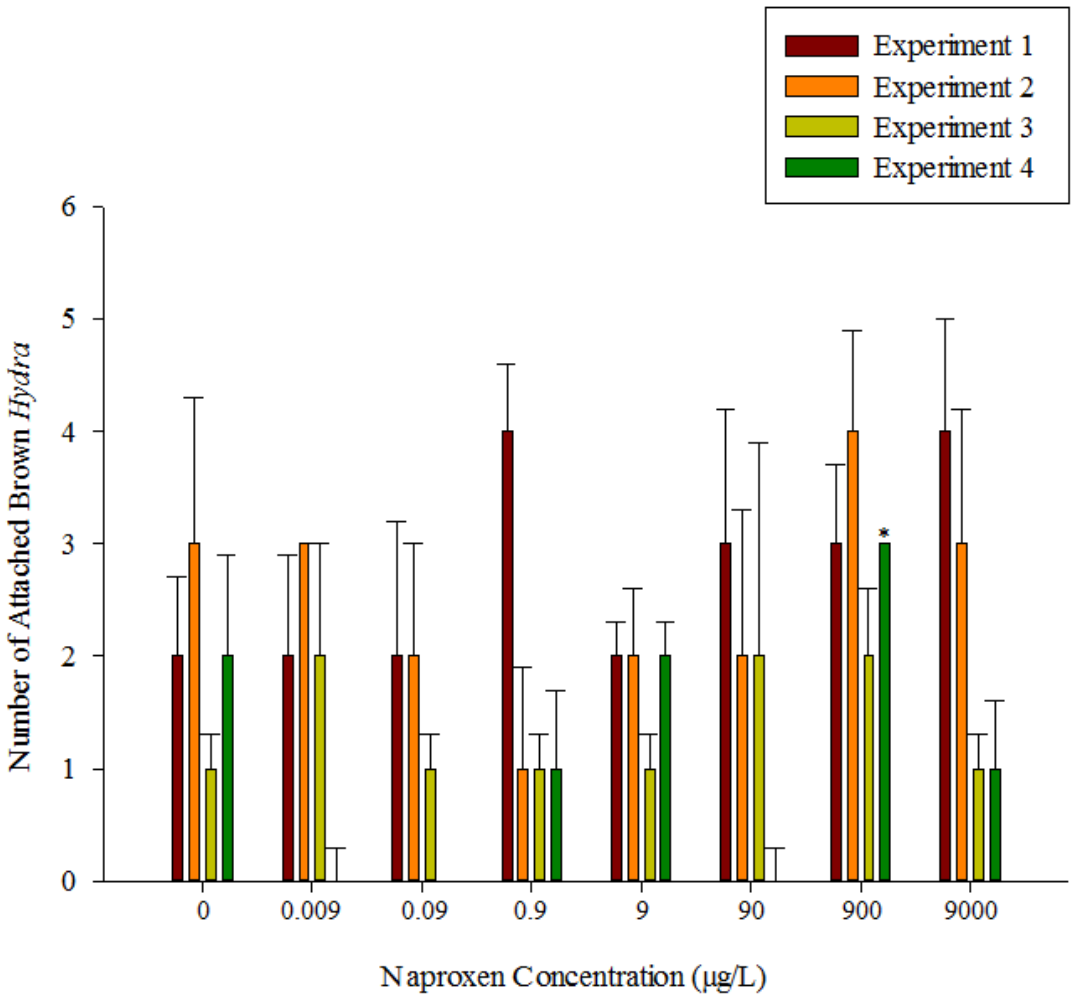


Figure 11: **Average number of attached brown *Hydra* in experiments 1 to 4 after 7 days of exposure to various concentrations of naproxen.** Attachment of brown *Hydra* was significantly higher at 900 µg/L compared to 90 µg/L of naproxen in the fourth experiment, determined through *Post Hoc* analysis (*p = 0.03). The solid bars represent the average value found for 3 wells (total of 15 *Hydra*) per concentration and the error bars represent standard error. A positive control (copper) was run during each experiment to ensure that *Hydra* behaved in a predicted and previously documented way. Any solid bars that are not shown represent the average number of attached *Hydra* is zero for that experiment.

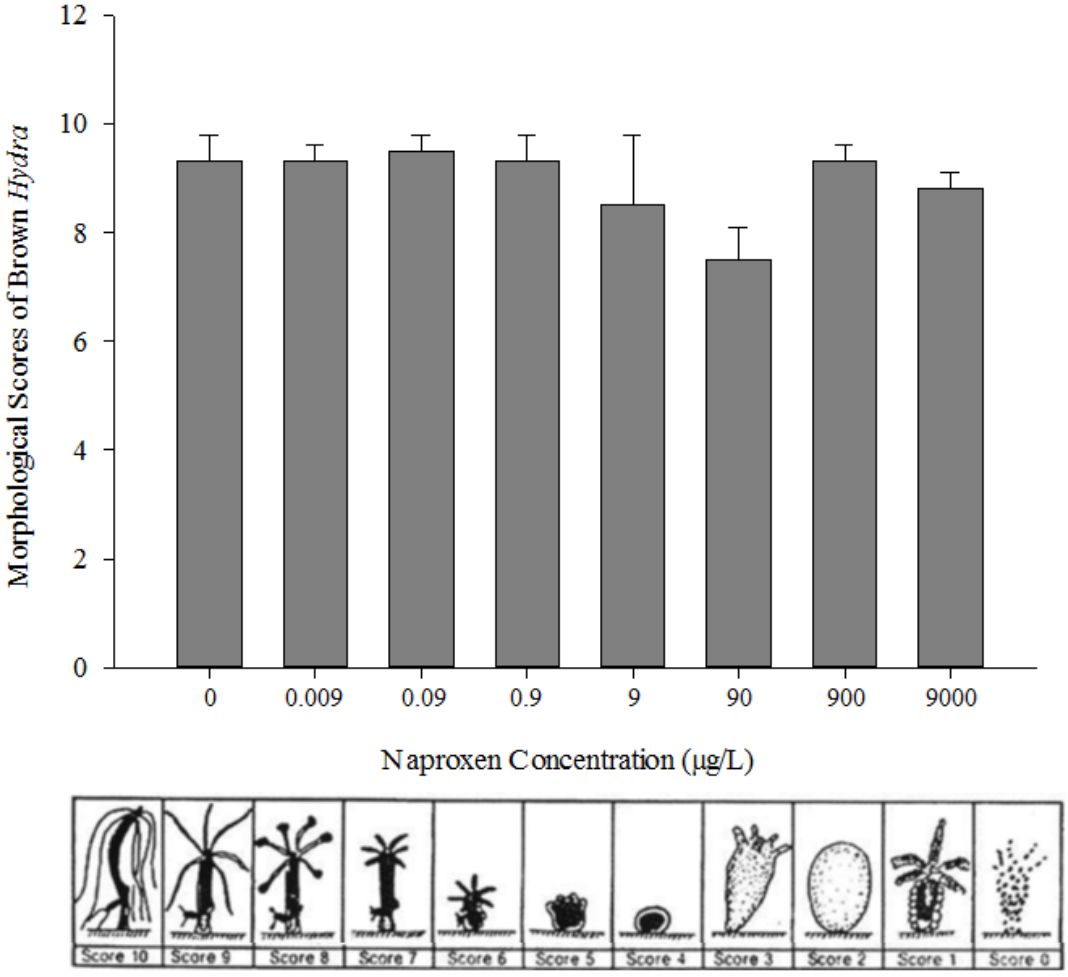


Figure 12: **Average morphological scores of brown *Hydra* in experiments 1 to 4 were pooled after 7 days of exposure to various concentrations of naproxen.** No statistically significant results were found. The solid bars represent the average value found for 12 wells (total of 60 *Hydra*) per concentration and the error bars represent standard error. A positive control (copper) was run during each experiment to ensure that *Hydra* behaved in a predicted and previously documented way.

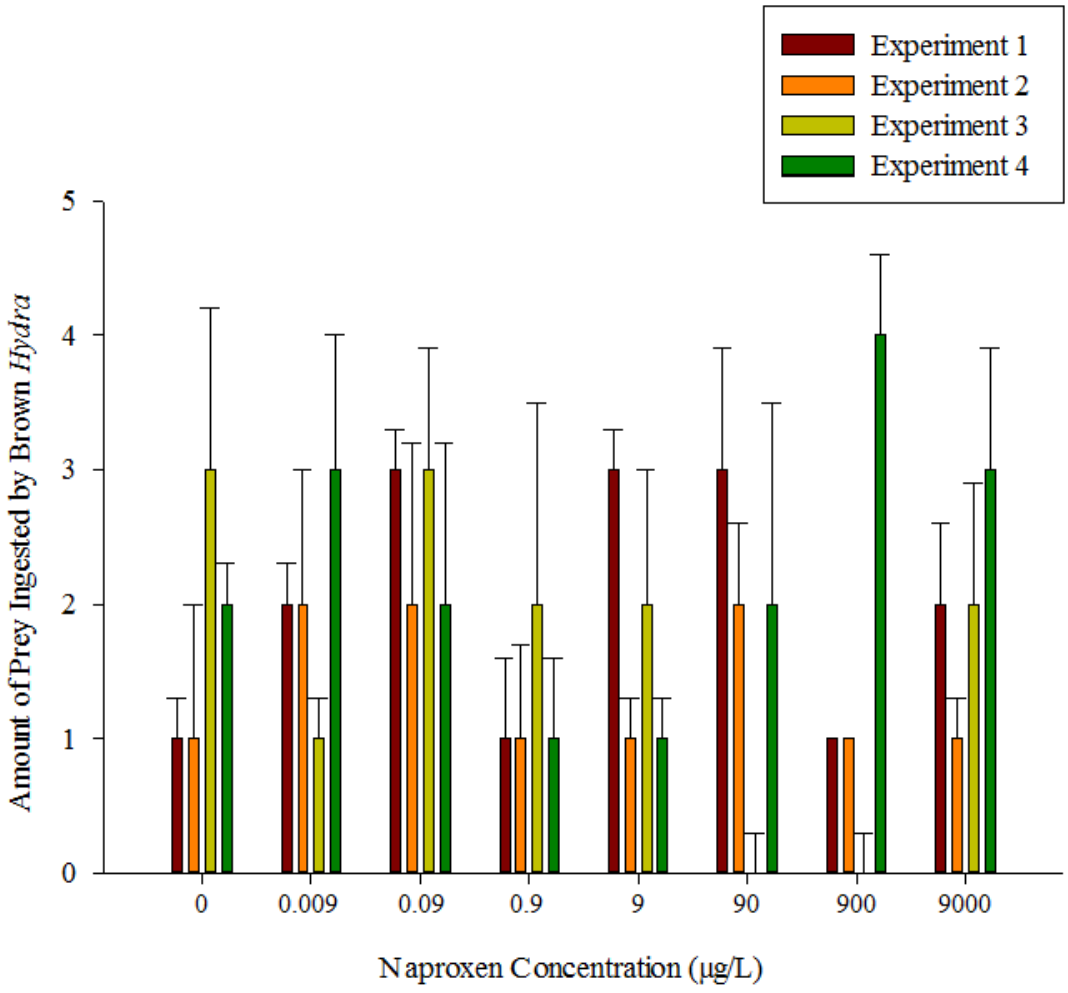


Figure 13: **Average number of prey ingested by brown *Hydra* 2 hours after 7 days of exposure to various concentrations of naproxen in experiments 1 to 4.** No statistically significant results were found. The solid bars represent the average value found for 3 wells (total of 15 *Hydra*) per concentration and the error bars represent standard error. A positive control (copper) was run during each experiment to ensure that *Hydra* behaved in a predicted and previously documented way. Any solid bars that are not shown represent the average number of prey ingested by *Hydra* is zero for that experiment.

Diclofenac

Attachment, morphology and prey ingested by green *Hydra* were evaluated in the four experiments run after 7 days of exposure to various concentrations of diclofenac (Appendix B: v). Attached green *Hydra* in experiments 1 to 4 were normally distributed and based on the results from a 2-way ANOVA, the experiments were pooled and analyzed together (Figure 14). Average number of attached green *Hydra,* using a 1-way ANOVA, was the same in all concentrations tested ($p = 0.09$, $F = 2.12$). No relationship was found between attached green *Hydra* and diclofenac when regression was used.

Morphology scores of green *Hydra* in experiments 1 to 4 were analyzed separately using non-parametric tests (Figure 15). Morphology scores of green *Hydra* in the second experiment, using Spearman Rank Correlation, showed a negative correlation with diclofenac (Spearman's $rho = -0.83$, $p = 0.01$).

Prey ingested by green *Hydra* 2 hours after 7 days of exposure to various concentrations of diclofenac in experiments 1 to 4 were normally distributed and based on the results from a 2-way ANOVA, the experiments were pooled and analyzed together (Figure 16). Average number of prey ingested by green *Hydra,* using a 1-way ANOVA, was the same in all concentrations tested ($p = 0.22$, $F = 1.49$). No relationship was found between prey ingested by green *Hydra* and diclofenac when regression was used.

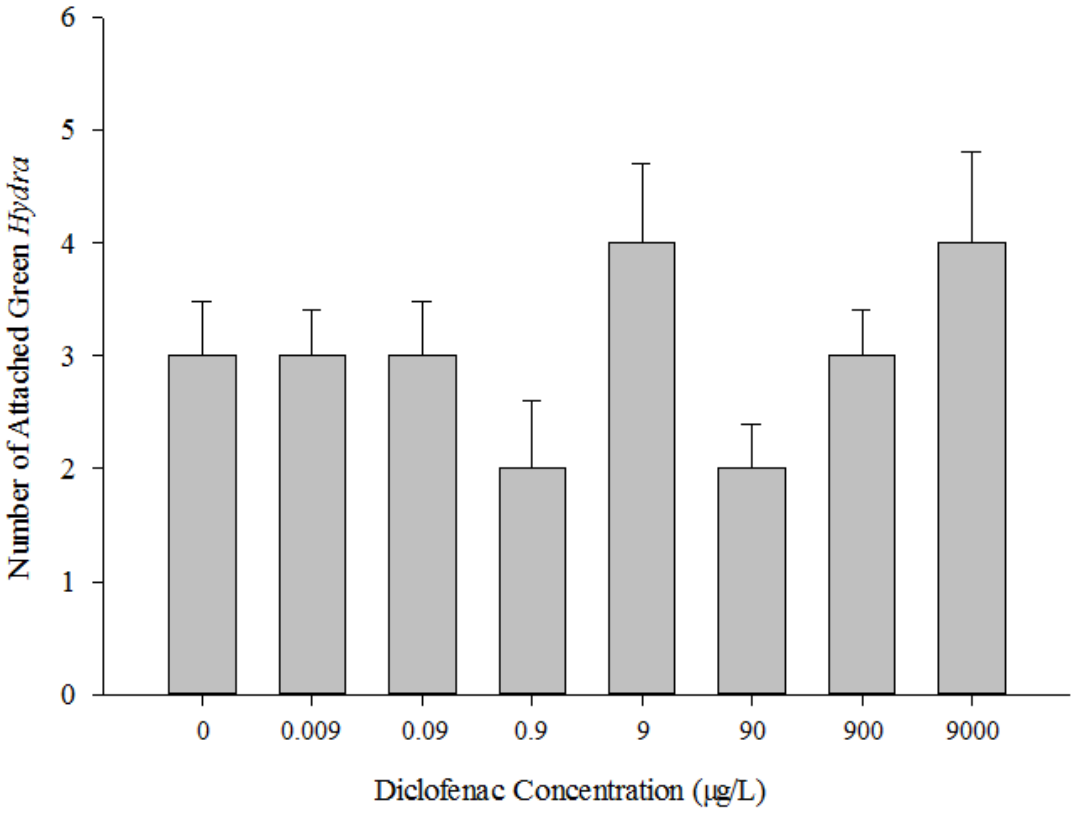


Figure 14: **Average number of attached green *Hydra* in experiments 1 to 4 were pooled after 7 days of exposure to various concentrations of diclofenac.** Average number of attached green *Hydra* was comparable in all concentrations tested ($p = 0.09$). The solid bars represent the average value found for 12 wells (total of 60 *Hydra*) per concentration and the error bars represent standard error. A positive control (copper) was run during each experiment to ensure that *Hydra* behaved in a predicted and previously documented way.

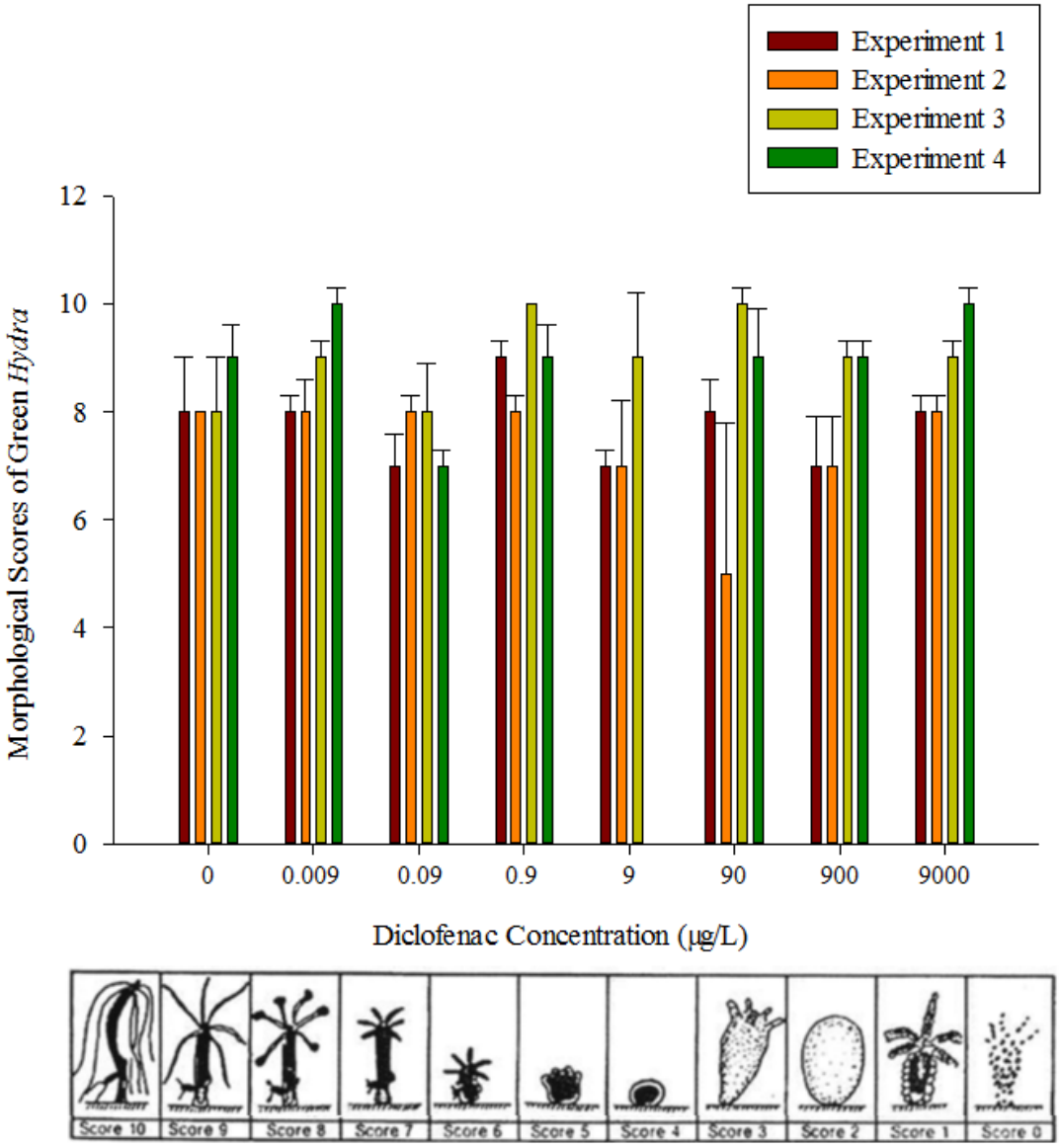


Figure 15: **Average morphology scores of green *Hydra* in experiments 1 to 4 after 7 days of exposure to various concentrations of diclofenac.** A negative correlation was found between morphology scores of green *Hydra* and diclofenac in the second experiment ($p = 0.01$). The solid bars represent the average value found for 3 wells (total of 15 *Hydra*) per concentration and the error bars represent standard error. A positive control (copper) was run during each experiment to ensure that *Hydra* behaved in a predicted and previously documented way. Any solid bars that are not shown represent an average morphology score of zero for that experiment.

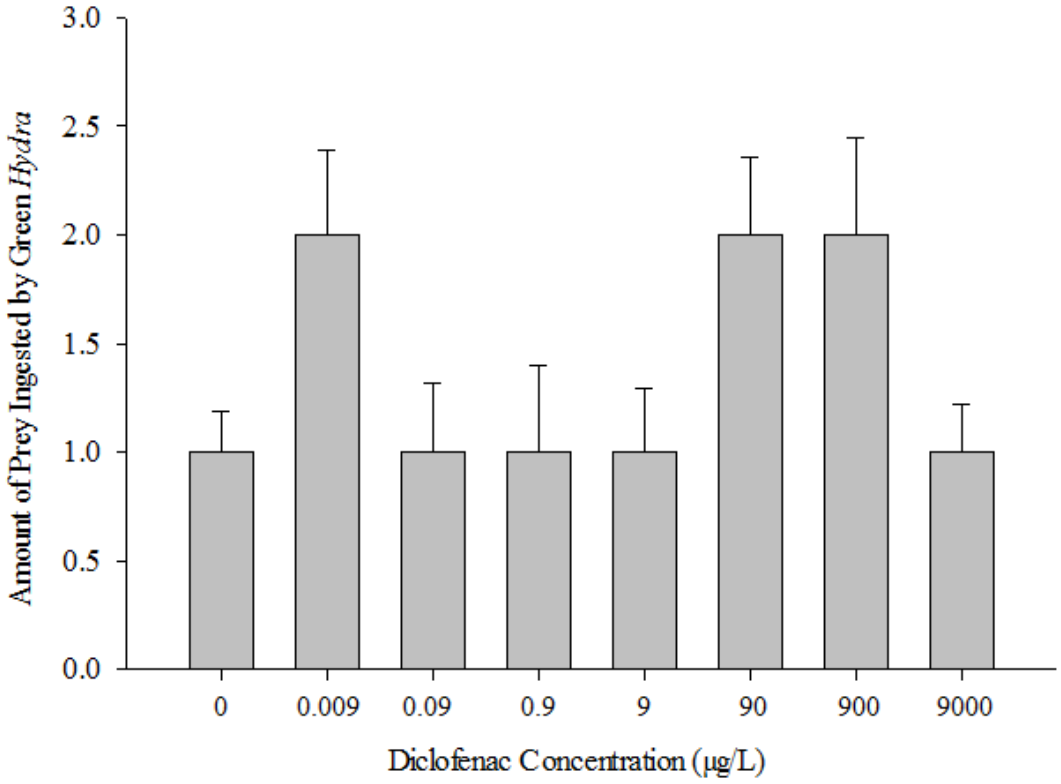


Figure 16: **Average number of prey ingested by green *Hydra* 2 hours after 7 days of exposure to various concentrations of diclofenac in experiments 1 to 4 were pooled.** Average amount of prey ingested by green *Hydra* was comparable in all concentrations tested ($p = 0.22$). The solid bars represent the average value found for 12 wells (total of 60 *Hydra*) per concentration and the error bars represent standard error. A positive control (copper) was run during each experiment to ensure that *Hydra* behaved in a predicted and previously documented way.

Attachment, morphology and prey ingested by brown *Hydra* were evaluated in the four experiments run after 7 days of exposure to various concentrations of diclofenac (Appendix B: vi). High mortality in control wells resulted in experiments 3 and 4 to be excluded from analysis. Attached brown *Hydra* in experiments 1 and 2 were normally distributed and based on a 2-way ANOVA, were analyzed separately (Figure 17). None of the experiments showed any statistically significant results.

Morphology scores of brown *Hydra* in experiments 1 to 2 were analyzed separately using non-parametric tests (Figure 18). None of the experiments showed any statistically significant results.

Prey ingested by brown *Hydra* in 2 hours after 7 days of exposure to various concentration of diclofenac in experiments 1 to 2 were normally distributed and based on the results from a 2-way ANOVA, the experiments were analyzed separately (Figure 19). None of the experiments showed any statistically significant results.

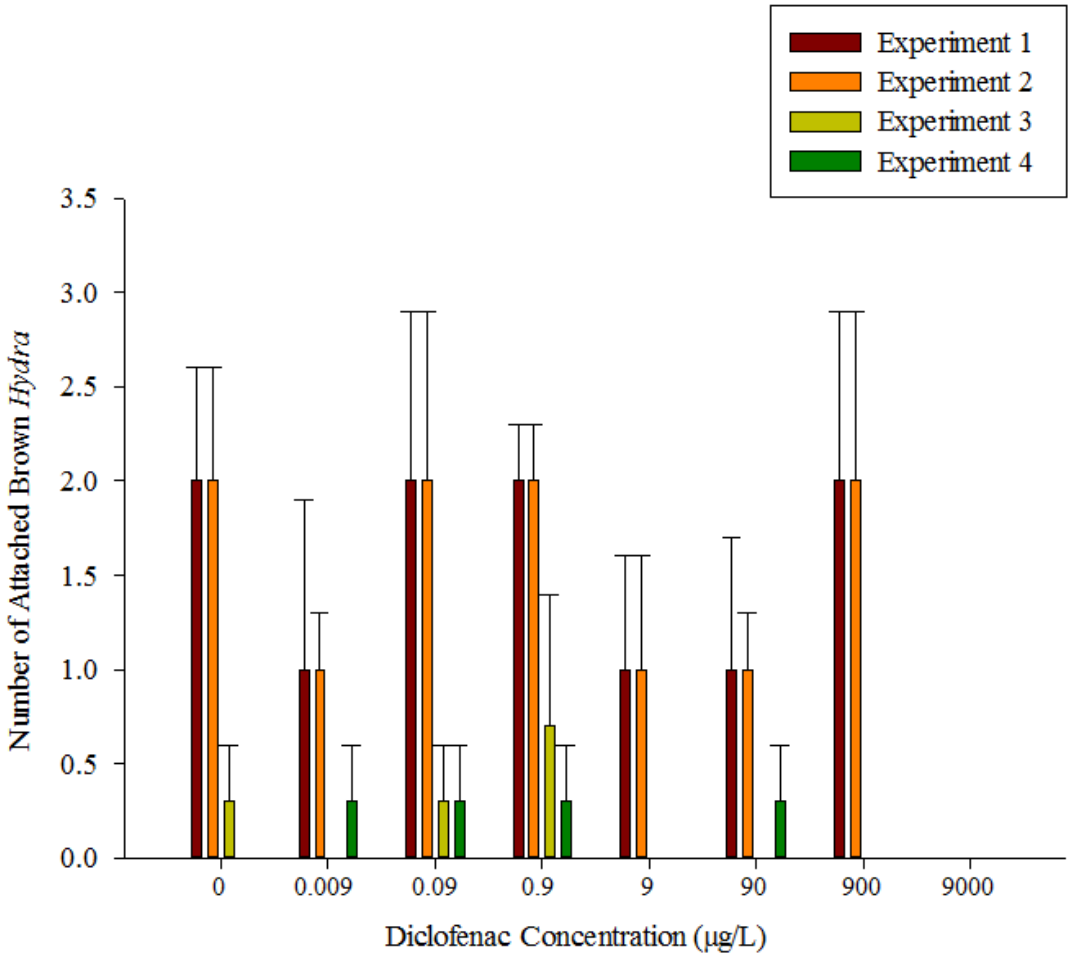


Figure 17: **Average number of attached brown *Hydra* in experiments 1 to 4 after 7 days of exposure to various concentrations of diclofenac.** No statistically significant results were found. The solid bars represent the average value found for 3 wells (total of 15 *Hydra*) per concentration and the error bars represent standard error. A positive control (copper) was run during each experiment to ensure that *Hydra* behaved in a predicted and previously documented way. Any solid bars that are not shown represent the average number of attached *Hydra* was zero for that experiment.

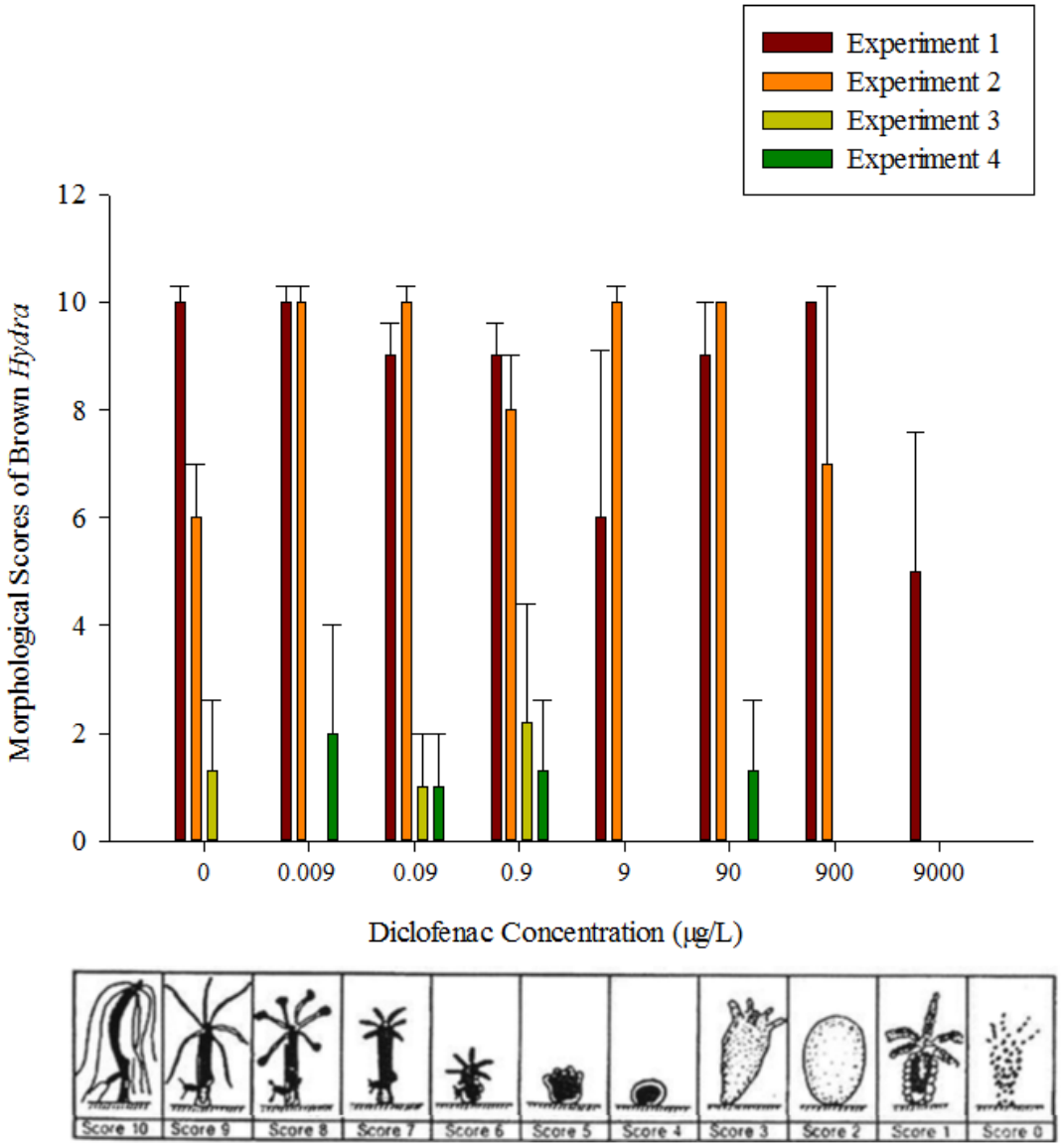


Figure 18: **Average morphology scores of brown *Hydra* in experiments 1 to 4 after 7 days of exposure to various concentrations of diclofenac.** No statistically significant results were found. The solid bars represent the average value found for 3 wells (total of 15 *Hydra*) per concentration and the error bars represent standard error. A positive control (copper) was run during each experiment to ensure that *Hydra* behaved in a predicted and previously documented way. Any solid bars that are not shown represent an average morphology score of zero for that experiment.

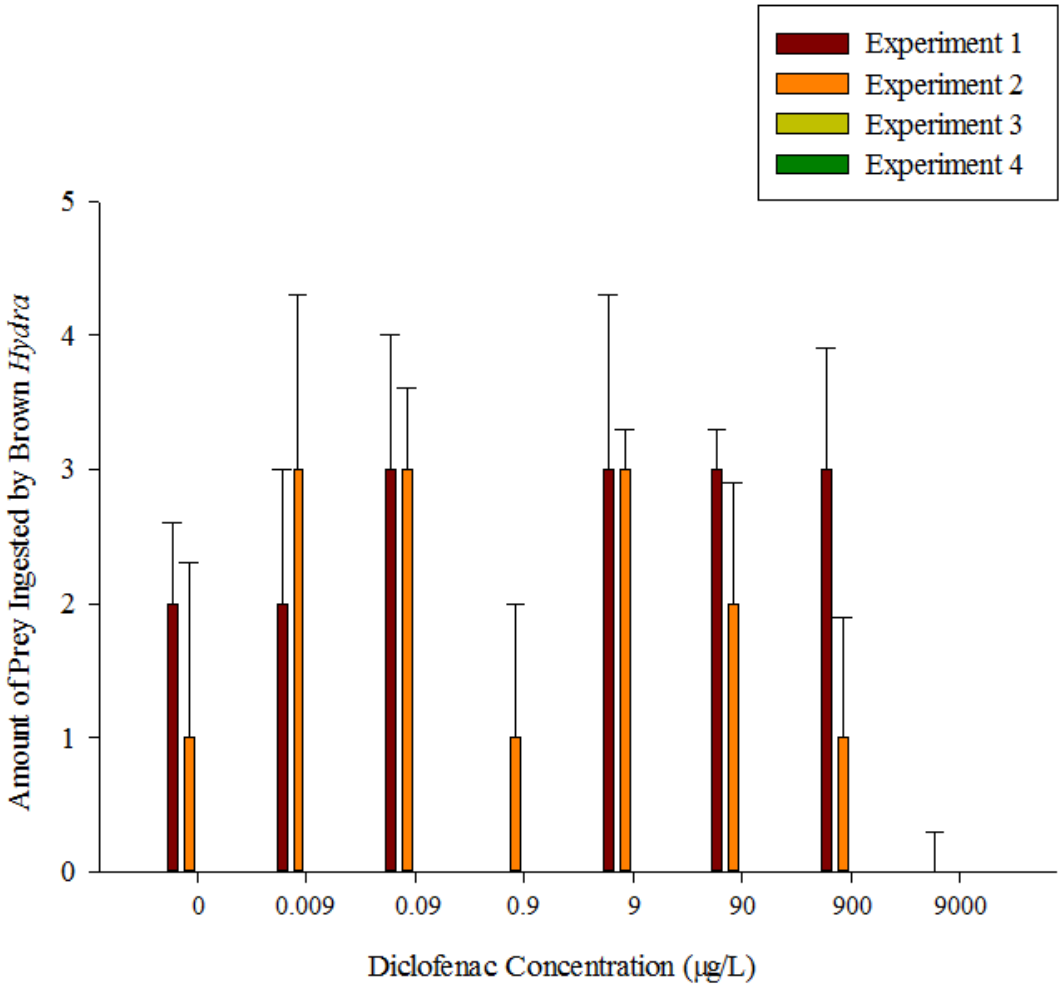


Figure 19: **Average number of prey ingested by brown *Hydra* 2 hours after 7 days of exposure to various concentrations of diclofenac in experiments 1 to 4.** No statistically significant results were found. The solid bars represent the average value found for 3 wells (total of 15 *Hydra*) per concentration and the error bars represent standard error. A positive control (copper) was run during each experiment to ensure that *Hydra* behaved in a predicted and previously documented way. Any solid bars that are not shown represent the average number of prey ingested by *Hydra* was zero for that experiment.

4.4 Population Reproduction Toxicity Test

Ibuprofen

Reproduction of green *Hydra* was evaluated after 7 days of exposure to various concentrations of ibuprofen (Appendix C: i & ii; Appendix D:i). Reproduction of green *Hydra*, using Spearman Rank Correlation, showed a negative correlation with ibuprofen (Spearman's rho = -0.51, p = 0.03) (Figure 20). The NOEC was determined to be 10,000 µg/L.

Reproduction of brown *Hydra* was evaluated after 7 days of exposure to various concentrations of ibuprofen (Appendix C: iii & iv; Appendix D: i). Reproduction of brown *Hydra*, using Spearman Rank Correlation, showed a negative correlation with ibuprofen (Spearman's rho = -0.58, p = 0.01) (Figure 20). The NOEC was determined to be 10,000 µg/L.

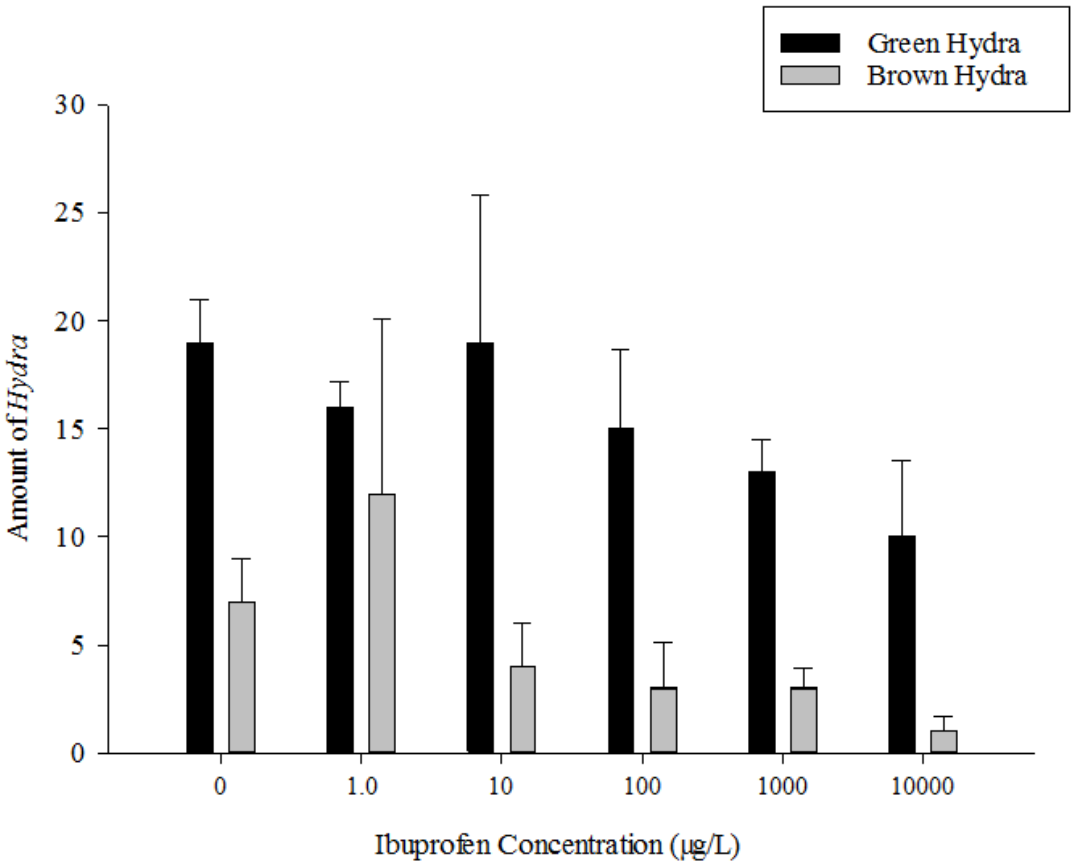


Figure 20: Average number of green and brown *Hydra* after a 7 day population reproduction test with various concentrations of ibuprofen. A negative correlation was found between reproduction of green and brown *Hydra* and ibuprofen ($p = 0.03$ and $p = 0.01$, respectively). The solid bars represent the average value found for 3 wells (total of 15 *Hydra*) per concentration and the error bars represent standard error.

Naproxen

Reproduction of green *Hydra* was evaluated after 7 days of exposure to various concentrations of naproxen (Appendix C: v & vi; Appendix D: ii). No relationship was found between reproduction of green *Hydra* and naproxen using Spearman Rank Correlation (Spearman's rho = 0.03, p = 0.92) (Figure 21). The NOEC was determined to be 9,000 µg/L.

Reproduction of brown *Hydra* was evaluated after 7 days of exposure to various concentrations of naproxen (Appendix C: vii & viii; Appendix D: ii). No relationship was found between reproduction of brown *Hydra* and naproxen using Spearman Rank Correlation (Spearman's rho = 0.21, p = 0.4) (Figure 21). The NOEC was determined to be 9,000 µg/L.

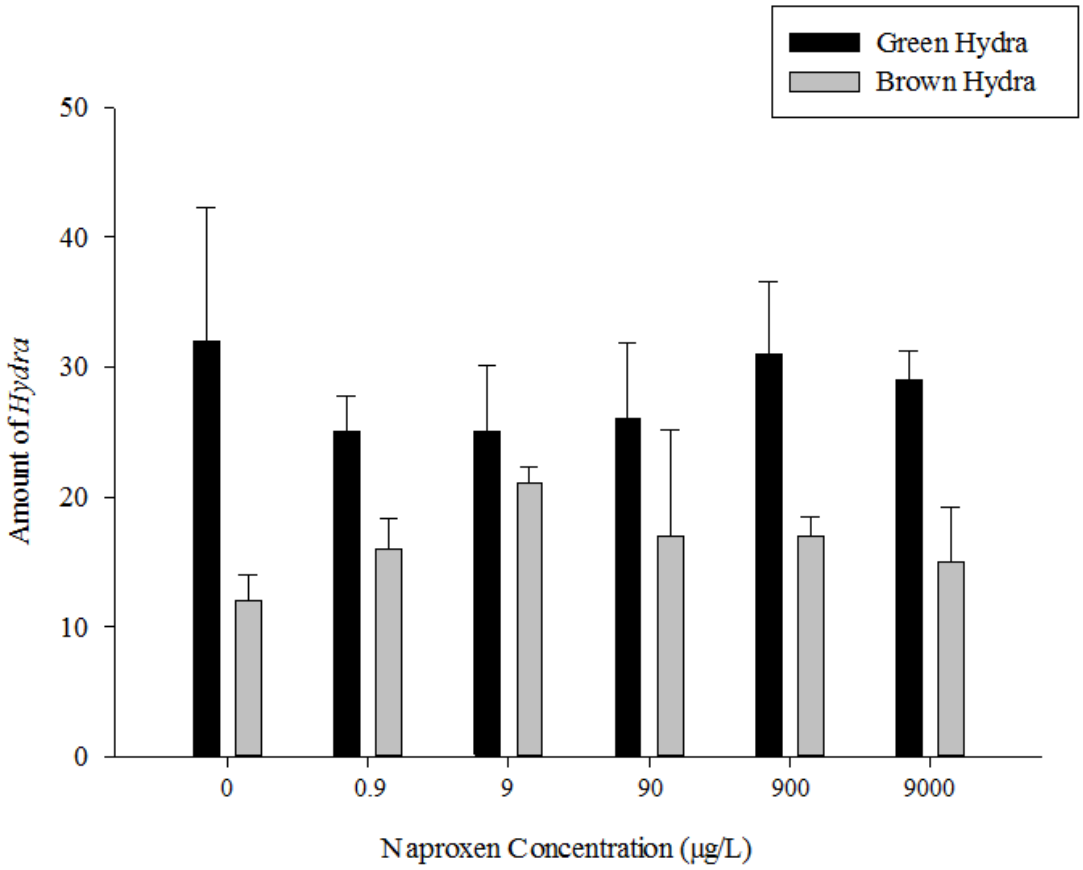


Figure 21: Average number of green and brown *Hydra* after a 7 day population reproduction test with various concentrations of naproxen. No statistically significant results were found. The solid bars represent the average value found for 3 wells (total of 15 *Hydra*) per concentration and the error bars represent standard error.

Diclofenac

Reproduction of green *Hydra* was evaluated after 7 days of exposure to various concentrations of diclofenac (Appendix C: ix – x; Appendix D: iii). No relationship was found between reproduction of green *Hydra* and diclofenac using Spearman Rank Correlation (Spearman's rho = -0.37, p = 0.14) (Figure 22). LOEC and NOEC values were determined to be 9,000 µg/L and 900 µg/L, respectively.

Reproduction of brown *Hydra* was evaluated after 7 days of exposure to various concentrations of diclofenac (Appendix C: xi & xii; Appendix D: iii). No relationship was found between reproduction of brown *Hydra* and diclofenac using Spearman Rank Correlation (Spearman's rho = -0.45, p = 0.06) (Figure 22). LOEC and NOEC values were determined to be 9,000 µg/L and 900 µg/L, respectively.

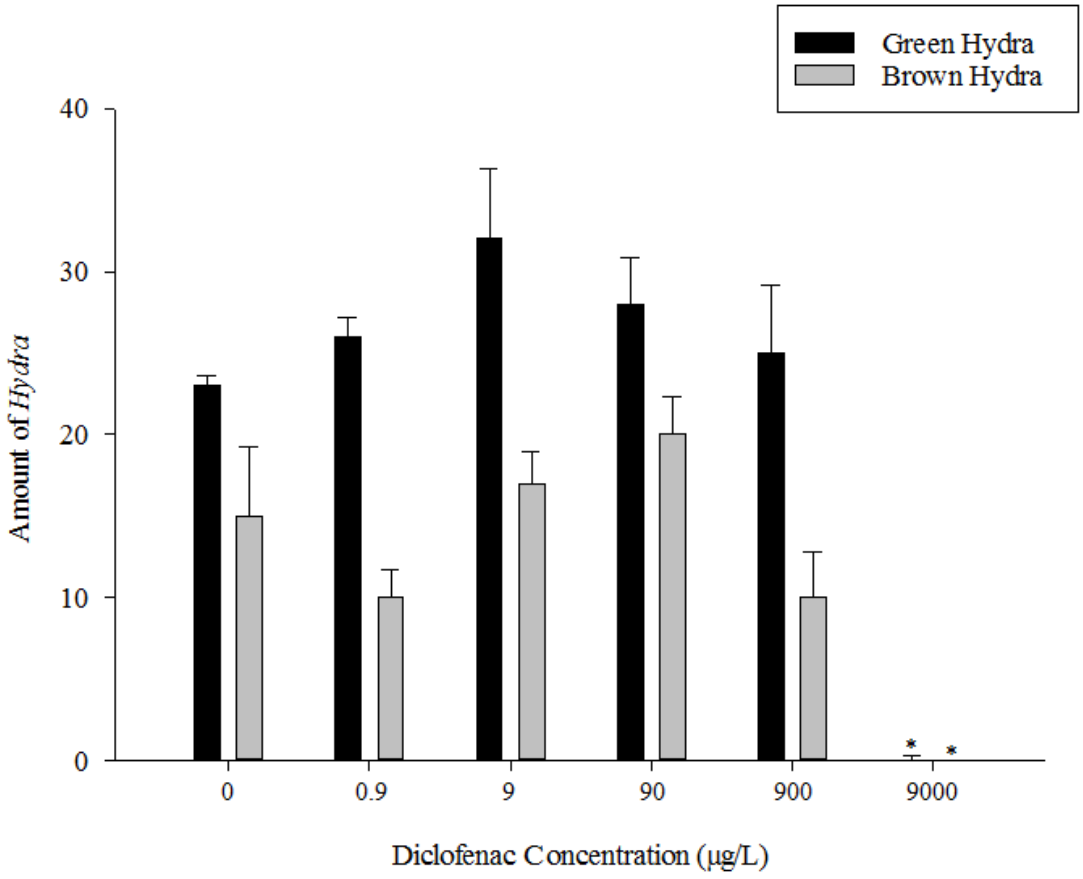


Figure 22: Average number of green and brown *Hydra* after a 7 day population reproduction test with various concentrations of diclofenac. *LOEC determined to be 9,000 µg/L for both species of *Hydra*. The solid bars represent the average value found for 3 wells (total of 15 *Hydra*) per concentration and the error bars represent standard error.

4.5 Species Comparison

Ibuprofen

Trends in attachment, morphology, prey ingested and reproduction of green and brown *Hydra* were compared after 7 days of exposure to various concentrations of ibuprofen. No relationship was found between attachment of green *Hydra* and ibuprofen. A negative correlation was found between attached brown *Hydra* and ibuprofen in the second experiment ($p = 0.04$). A positive correlation was found between attached brown *Hydra* and ibuprofen in the fourth experiment ($p = 0.04$). No relationship was found between morphology and prey ingested by green *Hydra* and ibuprofen. A negative correlation was found between morphology scores of brown *Hydra* and ibuprofen in the second experiment ($p = 0.04$). A negative correlation was found between the amount of prey ingested by brown *Hydra* 2 hours after exposure to ibuprofen in the second experiment ($p = 0.04$). Reproduction of green and brown *Hydra* cannot be directly compared because the tests were performed under different environmental conditions (temperature control room and laboratory bench). However, both species showed a negative correlation between reproduction and ibuprofen ($p = 0.03$, $p = 0.01$, respectively). NOEC values found were the same for both species.

Naproxen

Trends in attachment, morphology, prey ingested and reproduction of green and brown *Hydra* were compared after 7 days of exposure to various concentrations of naproxen. No relationship was found between attachment, prey ingested and reproduction by green and brown *Hydra* and naproxen. A negative correlation was found between morphology scores of green *Hydra* and naproxen in the fourth experiment ($p = 0.04$). No relationship was found between morphology scores of brown *Hydra* and naproxen. NOEC values were the same for both species of *Hydra.*

Diclofenac

Trends in attachment, morphology, prey ingested and reproduction of green and brown *Hydra* were compared after 7 days of exposure to various concentrations of diclofenac. No relationship was found between attachment, prey ingested and reproduction by green and brown *Hydra* and diclofenac. A negative correlation was found between morphology scores of green *Hydra* and diclofenac in the second experiment ($p = 0.01$). No relationship was found between morphology scores of brown *Hydra* and diclofenac. LOEC and NOEC values were the same for both species; 9,000 µg/L and 900 µg/L, respectively.

Section 5: Discussion

5.1 Summary and Interpretation of Results

Do select NSAIDs cause chronic toxicity in *Hydra*? Is there a difference in NSAID toxicity between different species of *Hydra*? These questions were addressed using 7 day exposure toxicity tests and population reproduction tests with 2 species of *Hydra.* Several negative correlations were shown between ibuprofen, naproxen, diclofenac and *Hydra.* However, these correlations generally occurred in only 25% or less of the 7 day exposure toxicity tests performed. Only 25 to 50% of these experiments showed differences between species in the endpoints evaluated.

Positive Control

Copper is a known toxicant to *Hydra,* however, differences between species was observed. Brown *Hydra* were found to have a higher tolerance for copper than green *Hydra.* This trend was expected as Källqvist & Meadows (1978) showed that copper can behave as an algal toxicant, affecting the algal symbiots found in the green *Hydra.* This finding suggests that the algal symbiosis could increase the toxicity that certain xenobiotics, such as metals, have on *Hydra* survival and morphology.

Attachment

Attachment of green *Hydra* was significantly higher at 10,000 µg/L in the third green *Hydra* experiment compared to all other concentrations tested ($p = 0.01$). Due to increased reproduction, the number of attached green *Hydra* found was almost double the number of *Hydra* originally placed into those wells. Comparing across the 7 day toxicity tests performed, this was the only experiment that showed an average increase in the number of *Hydra* that was greater than 1. A low average increase in *Hydra* number over the duration of the tests was

expected, this is due to the starving conditions the *Hydra* were maintained in. If feeding was introduced into the protocol during the 7 day exposure, such results might be altered since lack of food would no longer be a stressor. The starvation conditions were utilized based on a review of previously published protocols.

A negative correlation was found between attached brown *Hydra* and ibuprofen in the second brown *Hydra* experiment ($p = 0.04$). A positive correlation was found between attached brown *Hydra* and ibuprofen in the fourth experiment ($p = 0.04$). Possible explanation for these results could be due to the variation found in the controls. A large variation in responses with an alpha value of 0.05 could show false correlations. Further studies utilizing this endpoint should involve an increase in alpha value as well as increasing the sample size to minimize the effects of the inherent experimental variation.

No experiments in any of the 7 day exposure toxicity tests with naproxen or diclofenac showed a relationship between pharmaceutical exposure and attachment. A decline in attached brown *Hydra* was found to occur at 10,000 µg/L of naproxen after 96 hours of exposure (Quinn *et al.,* 2008b). This value exceeds the range of naproxen tested in this study; the highest concentration tested was 9,000 µg/L. Attachment occurs in *Hydra* when secretory granules, located in the basal disc, come in contact with a substrate and attach (Chaet, 1965). More studies would be needed to see if NSAIDs affect these secretory granules. In humans, NSAIDs have been found to affect ATP-sensitive potassium channels in beta cells that control secretion of insulin (Li *et al.,* 2007). If the secretions occur in the same or similar channels in *Hydra,* perhaps attachment could be affected using this mechanism by NSAIDs at high enough concentrations.

Morphology

A negative correlation was found between morphology scores of brown *Hydra* and ibuprofen in the second experiment ($p = 0.04$). A negative correlation was also found between morphology scores of green *Hydra* with both naproxen and diclofenac in the fourth and second experiments, respectively ($p=0.04$, $p = 0.01$). Morphology of brown *Hydra* has previously been found to have a negative relationship with ibupprofen and naproxen (Quinn *et al.,* 2008a; Quinn *et al.,* 2009). Such species differences in sensitivity could possibly be due to the absence or presence of algal symbiosis. Green *Hydra* seemed to be less tolerant to naproxen and diclofenac than brown *Hydra* in terms of morphology. The algal symbiots are mostly located in the upper body column. Thus, as morphology is impacted in this region, restriction in any advantages that the algal symbiosis might provide could occur (Habetha *et al.,* 2003). Even though this endpoint was blinded, some subjectivity still existed between scores that were morphologically close together. Creation of an unbiased systematic approach to morphology might alleviate this problem. A possible future avenue to explore could be examining if any biochemical markers change with changes in *Hydra* morphology.

Prey Ingestion

A negative correlation was found between the number of prey ingested by brown *Hydra* 2 hours after exposure to ibuprofen in the second experiment ($p = 0.04$). The impact of ibuprofen on prey ingestion has been inconsistent in the literature. Studies have shown that prey ingestion can have both a positive and negative relationship with ibuprofen (Pascoe *et al.,* 2003; Quinn *et al.,* 2008a; Quinn *et al.,* 2009). With an endpoint like prey ingestion, assumptions on the outcome of the controls are difficult to make. It should not be assumed that after the specific time period assigned, the control *Hydra* will eat all available prey. Unpredictable controls present a

problem when trying to determine if the differences between treatments is due to the presence of the xenobiotic being studied.

Reproduction

The only correlation that was found with population reproduction occurred with an ibuprofen exposure in both green and brown *Hydra* ($p = 0.03$, $p = 0.01$). There are no comparative studies in the literature that examine the effect of ibuprofen, naproxen, nor diclofenac on population reproduction of *Hydra.* However, a decrease in brown *Hydra* numbers was found to occur at ibuprofen and naproxen exposure concentrations of 5,000 and 11,000 µg/L, respectively (Quinn *et al.,* 2008a). Therefore, the negative correlation found between ibuprofen and *Hydra* population reproduction observed in this study could be expected if this concentration affects *Hydra* survival. One possible explanation why naproxen exposure did not affect *Hydra* population reproduction in this study could be related to the concentration tested being below those previously used.

The NOEC values for ibuprofen and naproxen for both species of *Hydra* was determined to 10,000 and 9,000 µg/L, respectively. The LOEC and NOEC values in this study for diclofenac for both species of *Hydra* were determined to be 9,000 µg/L and 900 µg/L, respectively. The 96 hour LOEC and NOEC for ibuprofen based on changes in morphology in brown *Hydra* was previously reported to be 1,000 µg/L and 100 µg/L, respectively (Quinn *et al.,* 2008b). The naproxen 96 hour LOEC and NOEC based on changes in morphology in brown *Hydra* was previously reported to be 5,000 and 1,000 µg/L, respectively (Quinn *et al.,* 2008b).

In Canada, ibuprofen, naproxen and diclofenac have been found in sewage effluent and river waters at concentrations ranging from 0.01 to 85 µg/L (Stumpf *et al.,* 1996; Ternes, 1998; Metcalfe *et al.,* 2003; Weigal *et al.,* 2004; Gagne *et al.,* 2006). Based on the LOEC and NOEC

values determined in this study along with those previously reported in the literature, NSAIDs at these environmentally relevant concentrations should not negatively impact *Hydra* populations. However, this study only examined the parent compounds in isolation and further research should be done in mixture studies as well as using photoproducts in order to provide a more accurate measure of potential toxicity.

5.2 Habitats of Hydra

Hydra's ecological habitats are another factor that makes these organisms excellent candidates for toxicity testing using effluent pharmaceuticals. Green *Hydra* inhabit waters which would be exposed to photoproducts of effluent pharmaceuticals since the surface region of lakes, where they live, are exposed to such large amounts of sunlight. Photoproducts of some specific drugs are more toxic than their parent compounds (Isidon *et al.,* 2005). Brown *Hydra* live in a more benthic region which would be exposed to those effluent pharmaceuticals found near or in the sediment.

Dissolved oxygen levels vary between aquatic ecosystem zones. Pharmaceuticals exposed to different amounts of oxygen could make these drugs more hydrophilic, possibly changing their toxicity. Since *Hydra* are found in both the lowest and highest oxygen containing regions, they could allow for differences in toxicity based on physical location.

Different preferences for water flow between species might provide another reason why *Hydra* would make good candidates for effluent pharmaceutical toxicity testing. Brown *Hydra* prefer flowing water which allows for oxygen replacement and renewal in the lower oxygen content zone they occupy. Green *Hydra* can live in slow moving water because the algal symbiosis provides the *Hydra* with additional oxygen through photosynthesis.

Lastly, *Hydra* are diploblastic. This unique feature allows for all the cells of the *Hydra* to be directly in contact with the exposed xenobiotic. This creates a high surface area to volume ratio that can thus increase absorption and thereby changes both the bioavailability and the subsequent toxicity of the drug. Having all cells directly exposed to their environment thus provides *Hydra* with an additional advantage as a potential toxicity test organism.

5.3 Strengths and Limitations

Hydra are considered to be ageless and *Hydra* can reproduce asexually by budding within 1 to 4 days (Loomis & Lenhoff, 1956). Therefore, using an exposure period that exceeds the time needed for *Hydra* to reproduce is a useful approach to assess chronic toxicity of contaminants. The possibility of species differences in *Hydra* toxicity has not been extensively addressed in literature previously. Additionally, the solvent carriers previously used in assessing NSAID toxicity have been found to be toxic to *Hydra* (Pascoe *et al.,* 2003; Quinn *et al.*, 2008ab; Quinn *et al.,* 2009). In this research, laboratory water was used as the rearing solution, in the negative controls, as well as the dilution medium used to create the concentrations tested. The lack of a solvent carrier thus did not inhibit survival nor reproduction of the *Hydra* tested. This strengthens the results found in this study as well as making them more environmentally relevant. A positive control (copper sulfate) was also used with every 7 day toxicity test which ensured that any changes in overall sensitivity of *Hydra* over time were detected.

Some limitations of this study include the large variations observed in controls with some of the endpoints studied. The chosen endpoints can be very robust. This might explain why attachment is not commonly used in literature. Another limitation to this study was that the selected pharmaceuticals were tested individually. Effluent pharmaceuticals enter aquatic

environments as complex mixtures, which have been documented to sometimes increase their toxicity to aquatic organisms (Quinn *et al.,* 2009).

Additionally, photoproducts have also been documented to be more toxic than the parent compounds (Isidon *et al.,* 2005). This study did not address how light or the presence of photoproducts could be affecting the toxicity of the selected NSAIDs.

Section 6: Conclusions

Several negative relationships were shown between ibuprofen, naproxen and diclofenac and *Hydra*. However, these relationships generally occurred in only one out of the four 7 day exposure toxicity tests performed. The selected NSAIDs, at environmentally revalent concentrations, thus do not appear to have significant chronic toxicity to green or brown *Hydra*. Differences in NSAID toxicity between different species of *Hydra* are unlikely. However, 25% to 50% of the experiments, showed some differences between species in the endpoints evaluated. Differences in tolerance or resistance of these drugs due to presence or absense of algal symbiots might be a possible explaination for these observed differences.

There are several conceivable next steps stemming from this research. The first could be creating mixtures of the selected NSAIDs and determining if the toxicity changes. If toxicity does change what would be the mechanism? Would additive, less-than-additive, antagonism or potentiation be observed?

One avenue to explore in the future would be the toxicity of photoproducts. Diclofenacs' photoproducts are reported to be up to 5-6 times more toxic than the parent compound and naproxen's photoproducts are also documented to be more toxic than the parent (Isidon *et al.*, 2005; Schmitt-Jansen *et al.*, 2007). Another potential approach could be to increase the concentration range tested to include those values found in literature that are known to cause negative effects. This would allow for discovery of why the endpoints are behaving in this manner. For example why would prey ingestion increase or decrease? Does glutathione conjugate with NSAIDs or is there another mechanism by which this takes place? The disadvantage of this approach, however, is that such concentrations greatly exceed those expected to be found in the environment.

The results found in this current study could help in providing a rationale for treating or removing pharmaceuticals by identifying that NSAIDs do not appear to be high risk pollutants to invertebrate populations. A long term goal of this research could be in the creation of a bioassay through the use of *Hydra* to test sewage effluent or wastewater for these types of pharmaceuticals. To conclude, current effluent concentrations of ibuprofen, naproxen and diclofenac are likely not negatively impacting the food web at this level, using *Hydr*a as a model invertebrate. Toxicity of these NSAIDs is also unlikely to significantly differ between the different species of *Hydra.*

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Section 8: Appendices

Appendix A: Positive control results using endpoints evaluated during 7 day exposure toxicity tests.

i: Positive control results using endpoints evaluated during 7 day exposure toxicity tests with ibuprofen. Correlations between the endpoints and copper are shown as either positive, negative or non-existent (-) using Spearman Rank Correlation.

Drug	Species	Endpoint	Exp. #1	Exp. #2	Exp. #3	Exp. #4
IBU	Green	Attachment	-	-	-	-
		Morphology	-	-	-	-
		Prey	-	-	-	-
	Brown	Attachment	-	Positive ($p = 0.01$)	-	-
		Morphology	-	Negative ($p = 0.04$)	-	-
		Prey	-	-	-	-

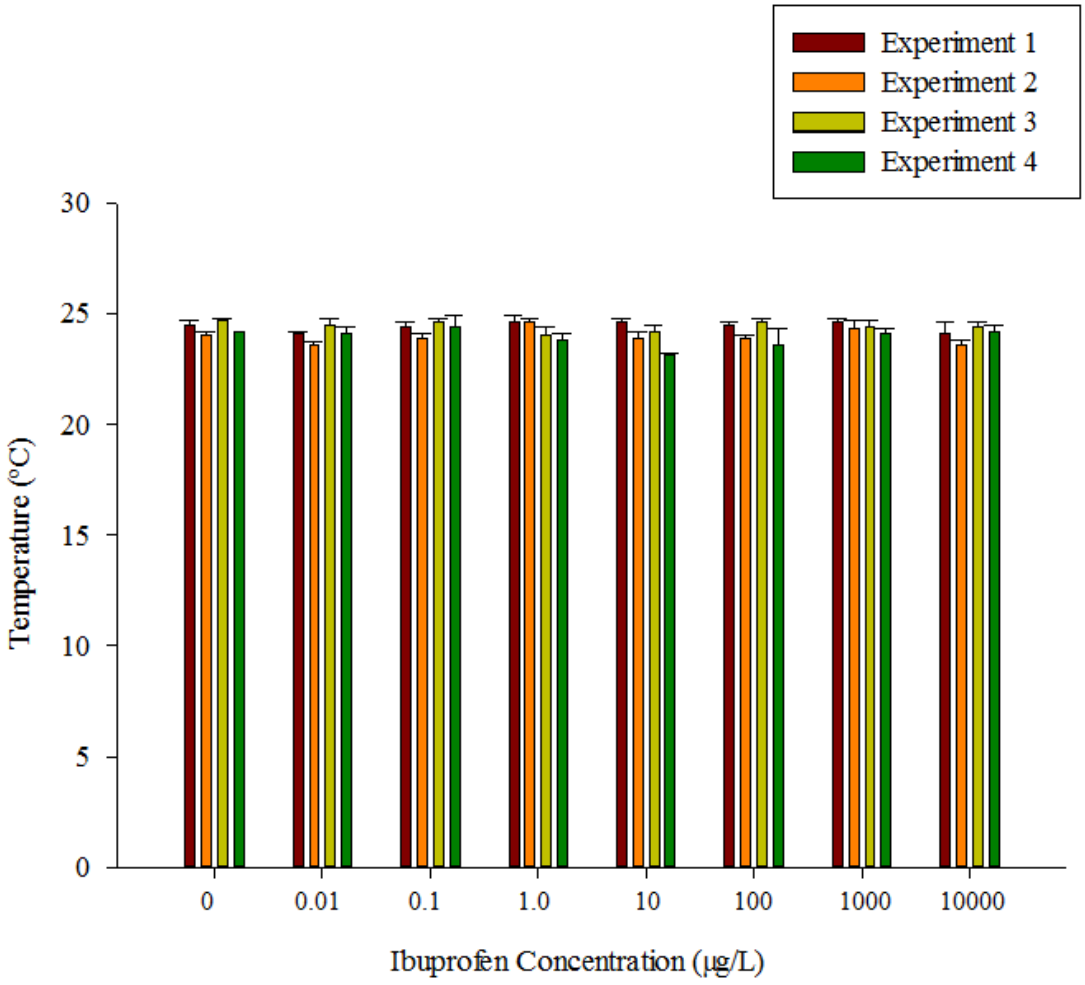
ii: Positive control results using endpoints evaluated during 7 day exposure toxicity tests with naproxen. Correlations between the endpoints and copper are shown as either positive, negative or non-existent (-) using Spearman Rank Correlation.

Drug	Species	Endpoint	Exp. #1	Exp. #2	Exp. #3	Exp. #4
NAP	Green	Attachment	Negative (P = 0.00)	Negative (P = 0.00)	-	-
		Morphology	Negative (P = 0.01)	Negative (P = 0.00)	Negative (P = 0.00)	-
		Prey	Negative (P = 0.01)	-	-	Negative (P = 0.00)
	Brown	Attachment	Negative (P = 0.00)	-	Positive (P = 0.02)	-
		Morphology	Negative (P = 0.00)	Negative (P = 0.01)	-	-
		Prey	Negative (P = 0.00)	-	-	-

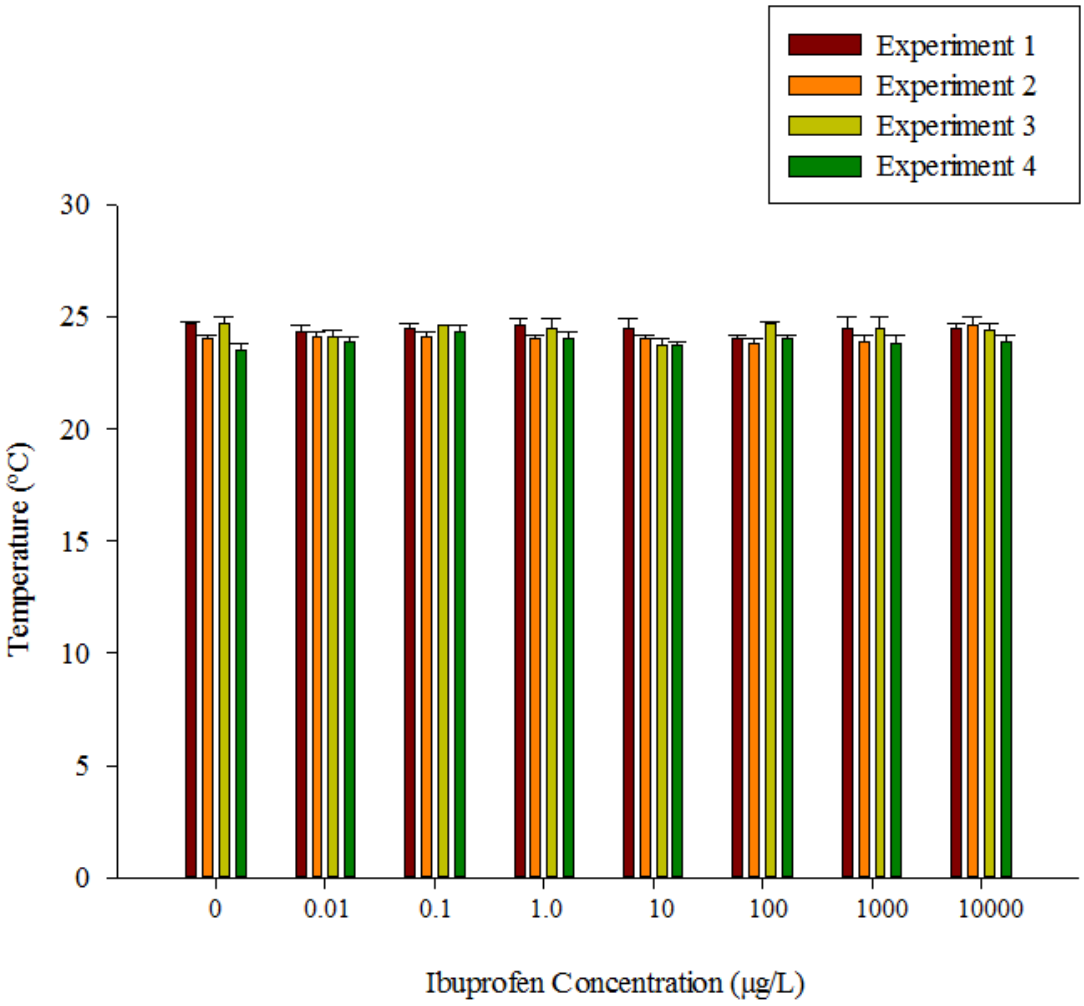
iii: Positive control results using endpoints evaluated during 7 day exposure toxicity tests with diclofenac. Correlations between the endpoints and copper are shown as either positive, negative or non-existent (-) using Spearman Rank Correlation.

Drug	Species	Endpoint	Exp. #1	Exp. #2	Exp. #3	Exp. #4
DICL	Green	Attachment	Negative (P = 0.00)	Negative (P = 0.01)	-	Negative (P = 0.00)
		Morphology	Negative (P = 0.00)	Negative P = (0.00)	-	Negative (P = 0.00)
		Prey	-	-	-	Negative (P = 0.01)
	Brown	Attachment	Negative (0.01)	Negative (0.01)	-	-
		Morphology	Negative (0.00)	Negative (0.00)	-	-
		Prey	Negative (0.01)	-	-	-

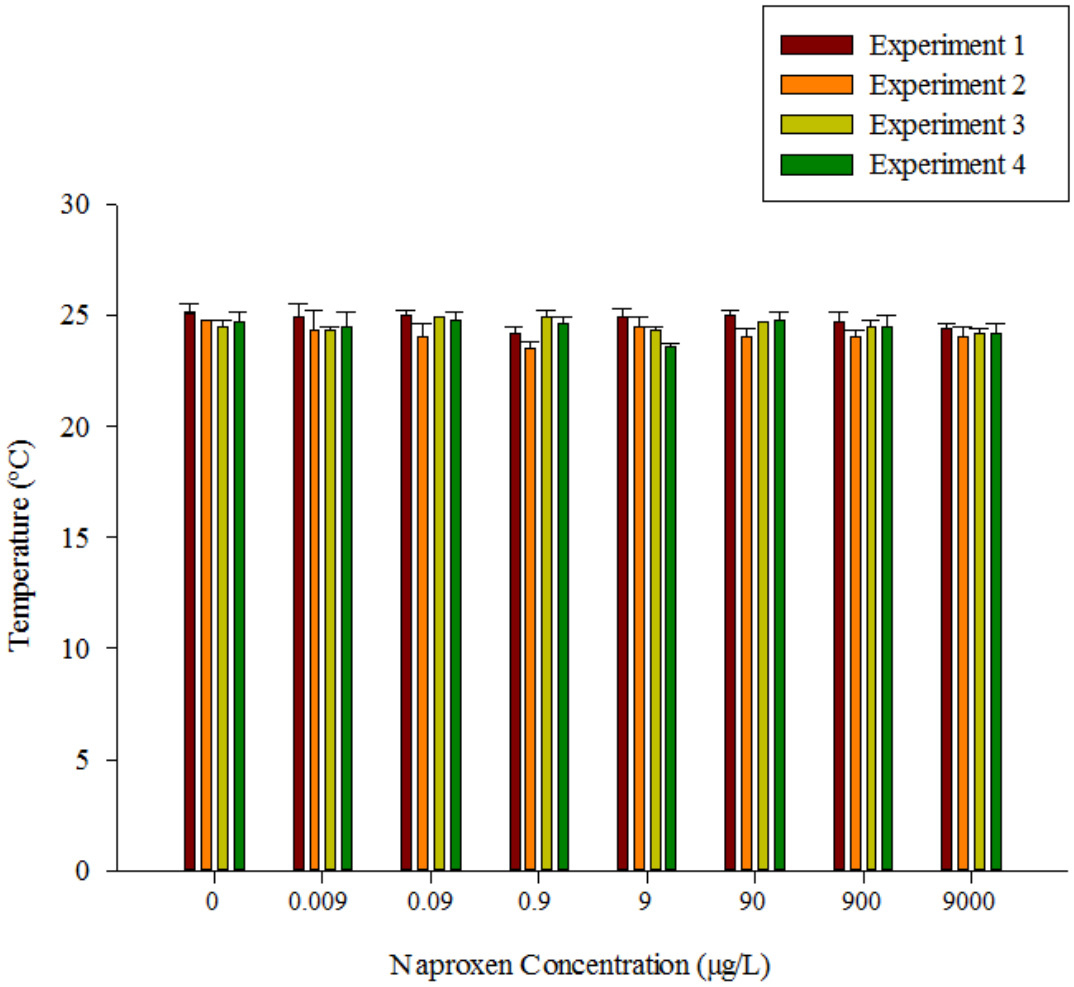
Appendix B: Average temperature (ºC) of green and brown *Hydra* after the 7 day exposure toxicity tests.



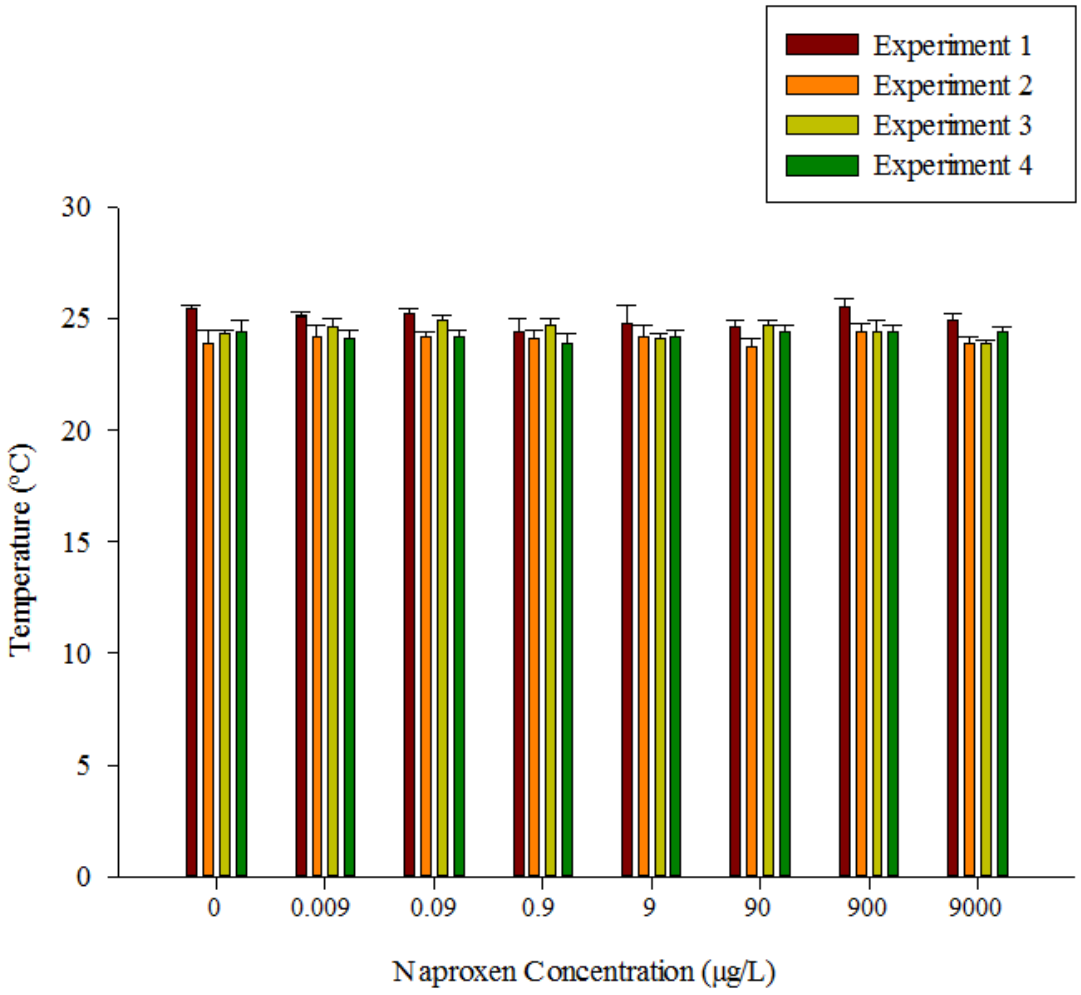
i: Average temperature (ºC) of green *Hydra* after the 7 day exposure toxicity tests with ibuprofen. The solid bars represent the average value found for 3 wells per concentration and the error bars represent standard error.



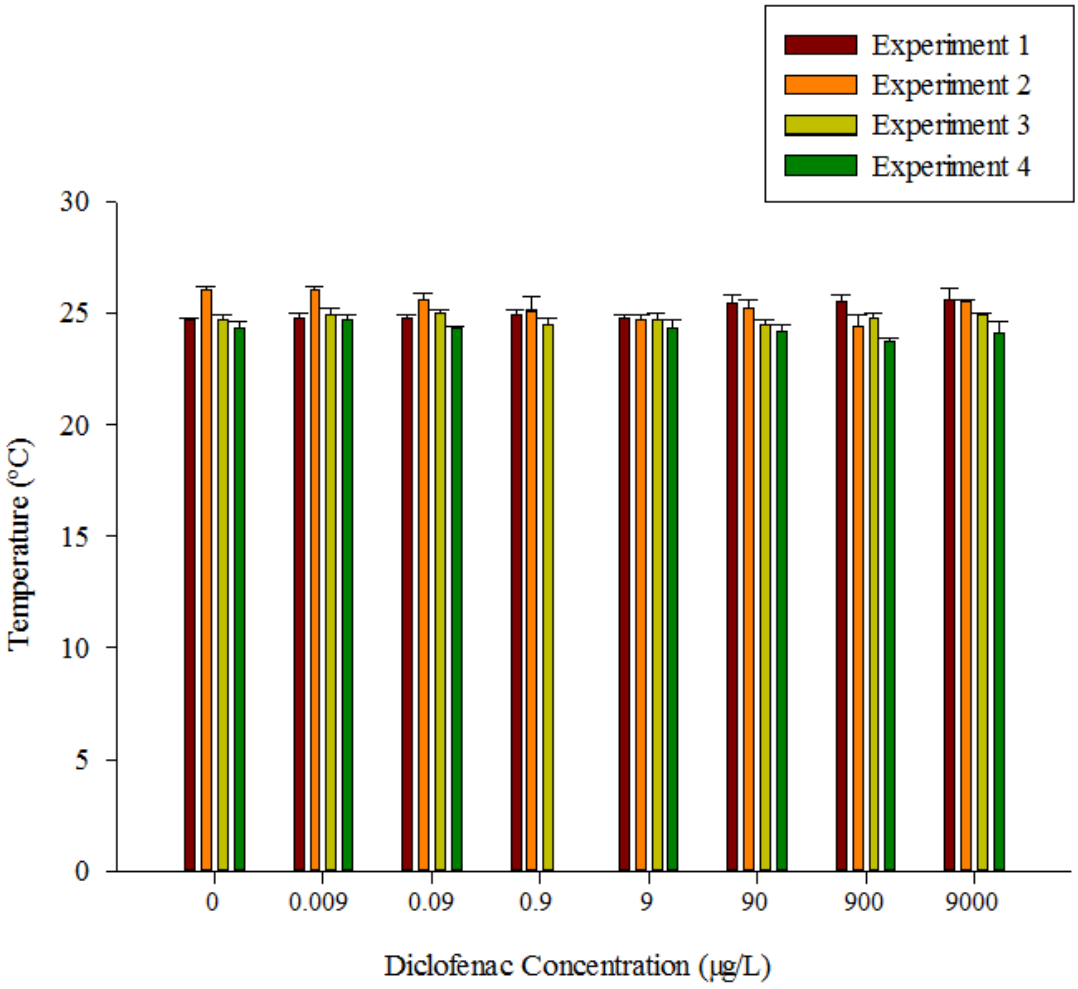
ii: Average temperature (°C) of brown *Hydra* after the 7 day exposure toxicity tests with ibuprofen. The solid bars represent the average value found for 3 wells per concentration and the error bars represent standard error.



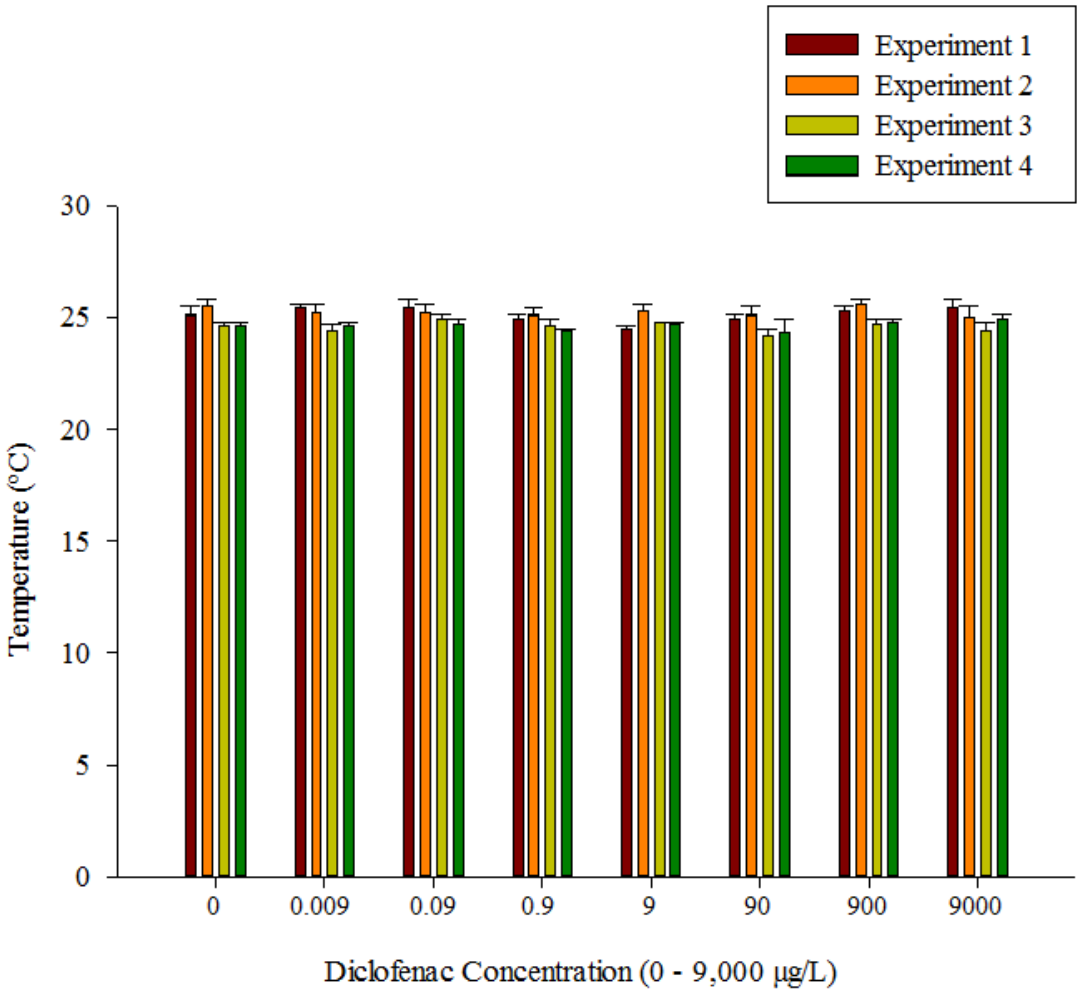
iii: Average temperature (°C) of green *Hydra* after the 7 day exposure toxicity tests with naproxen. The solid bars represent the average value found for 3 wells per concentration and the error bars represent standard error.



iv: Average temperature (°C) of brown *Hydra* after the 7 day exposure toxicity tests with naproxen. The solid bars represent the average value found for 3 wells per concentration and the error bars represent standard error.



v: Average temperature (°C) of green *Hydra* after the 7 day exposure toxicity tests with diclofenac. The solid bars represent the average value found for 3 wells per concentration and the error bars represent standard error.



vi: Average temperature (°C) of brown *Hydra* after the 7 day exposure toxicity tests with diclofenac. The solid bars represent the average value found for 3 wells per concentration and the error bars represent standard error.

Appendix C: Average rate of reproduction (k) of green and brown *Hydra* after the 7 day population reproduction tests.

i: Average number of green *Hydra* and rates of reproduction (k) after 7 days of exposure to various concentrations of ibuprofen. Rate of reproduction (k) was calculated by using the equation: $k = [\log_e(ny) - \log_e(nx)] / [ty - tx]$ where nx = number of hydra on first day and tx & ny = the number after y-x days (ty).

Concentration (µg/L)	**Number of *Hydra* Present**			**K Values**		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
0	15.0	21.0	21.0	0.16	0.21	0.21
1.0	15.0	14.0	18.0	0.16	0.15	0.18
10.0	13.0	12.0	33.0	0.14	0.13	0.27
100.0	8.00	19.0	19.0	0.07	0.19	0.19
1000.0	16.0	11.0	13.0	0.17	0.11	0.14
10000.0	7.00	17.0	6.00	0.05	0.17	0.03

ii: Average rate of reproduction and assigned critical values of green *Hydra* after 7 days of exposure to various concentrations of ibuprofen. The average rate of reproduction (mean k) is found for each concentration. The difference (d) from the control is then calculated by subtracting each mean k value by the mean k value found for the negative control. The d values collected for each concentration are then divided by the standard error (SE) calculated for each concentration tested (d/SE ratios). Next, a comparison of the d/SE ratios to critical values occurs by ordering the d/SE ratios (from smallest to largest) against a set of critical values (1.79, 1.96, 2.14 …). Any ordered d/SE ratio that exceeds their compared critical value is statistically significant. The LOEC is the lowest concentration that is statistically different from controls ($p \leq 0.05$) and the NOEC is the next lowest test concentration.

Concentration (µg/L)	**Mean K**	**d**	**d/SE**	**Critical Value**
0	0.57	0.00	0.00	0.00
1.0	0.49	0.08	0.07	2.14
10.0	0.53	0.04	0.00	1.79
100.0	0.45	0.12	0.03	1.96
1000.0	0.42	0.15	0.10	2.50
10000.0	0.25	0.32	0.09	2.32
*=Significantly different from control at $p \leq 0.05$				

iii: Average number of brown *Hydra* and rates of reproduction (k) after 7 days of exposure to various concentrations of ibuprofen. Rate of reproduction (k) was calculated by using the equation: $k = [\log_e(ny) - \log_e(nx)] / [ty - tx]$ where nx = number of hydra on first day and tx & ny = the number after y-x days (ty).

Concentration (µg/L)	**Number of *Hydra* Present**			**K Values**		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
0	3.00	7.00	10.00	-0.07	0.05	0.10
1.0	1.00	8.00	28.00	-0.23	0.07	0.25
10.0	2.00	2.00	8.00	-0.13	-0.13	0.07
100.0	0.00	2.00	7.00	0.00	-0.13	0.05
1000.0	2.00	3.00	5.00	-0.13	-0.07	0.00
10000.0	0.00	0.00	2.00	0.00	0.00	-0.13

iv: Average rate of reproduction and assigned critical values of brown *Hydra* after 7 days of exposure to various concentrations of ibuprofen. The average rate of reproduction (mean k) is found for each concentration. The difference (d) from the control is then calculated by subtracting each mean k value by the mean k value found for the negative control. The d values collected for each concentration are then divided by the standard error (SE) calculated for each concentration tested (d/SE ratios). Next, a comparison of the d/SE ratios to critical values occurs by ordering the d/SE ratios (from smallest to largest) against a set of critical values (1.79, 1.96, 2.14 …). Any ordered d/SE ratio that exceeds their compared critical value is statistically significant. The LOEC is the lowest concentration that is statistically different from controls ($p \leq 0.05$) and the NOEC is the next lowest test concentration.

Concentration (µg/L)	**Mean K**	**d**	**d/SE**	**Critical Value**
0	0.02	0.00	0.00	0.00
1.0	0.03	0.00	0.00	1.80
10.0	-0.06	0.09	0.04	2.14
100.0	-0.03	0.05	0.03	1.96
1000.0	-0.07	0.09	0.11	2.50
10000.0	-0.04	0.07	0.10	2.32
*=Significantly different from control at $p \leq 0.05$				

v: Average number of green *Hydra* and rates of reproduction (k) after 7 days of exposure to various concentrations of naproxen. Rate of reproduction (k) was calculated by using the equation: $k = [\log_e(ny) - \log_e(nx)] / [ty - tx]$ where nx = number of hydra on first day and tx & ny = the number after y-x days (ty).

Concentration (µg/L)	**Number of *Hydra* Present**			**K Values**		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
0.0	38.0	46.0	12.0	0.29	0.32	0.13
0.9	23.0	31.0	22.0	0.22	0.26	0.21
9.0	32.0	15.0	28.0	0.27	0.16	0.25
90.0	15.0	28.0	35.0	0.16	0.25	0.28
900.0	33.0	40.0	21.0	0.27	0.30	0.21
9000.0	33.0	27.0	26.0	0.27	0.24	0.24

vi: Average rate of reproduction and assigned critical values of green *Hydra* after 7 days of exposure to various concentrations of naproxen. The average rate of reproduction (mean k) is found for each concentration. The difference (d) from the control is then calculated by subtracting each mean k value by the mean k value found for the negative control. The d values collected for each concentration are then divided by the standard error (SE) calculated for each concentration tested (d/SE ratios). Next, a comparison of the d/SE ratios to critical values occurs by ordering the d/SE ratios (from smallest to largest) against a set of critical values (1.79, 1.96, 2.14 …). Any ordered d/SE ratio that exceeds their compared critical value is statistically significant. The LOEC is the lowest concentration that is statistically different from controls ($p \leq 0.05$) and the NOEC is the next lowest test concentration.

Concentration (µg/L)	**Mean K**	**d**	**d/SE**	**Critical Value**
0.0	0.24	0.00	0.00	0.00
0.9	0.23	0.01	0.00	2.50
9.0	0.22	0.02	0.00	2.32
90.0	0.23	0.02	0.00	2.14
900.0	0.26	-0.01	0.00	1.96
9000.0	0.25	0.00	0.00	1.79
*=Significantly different from control at $p \leq 0.05$				

vii: Average number of brown *Hydra* and rates of reproduction (k) after 7 days of exposure to various concentrations of naproxen. Rate of reproduction (k) was calculated by using the equation: $k = [\log_e(ny) – \log_e(nx)] / [ty – tx]$ where nx = number of hydra on first day and tx & ny = the number after y-x days (ty).

Concentration (µg/L)	**Number of *Hydra* Present**			**K Values**		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
0.0	14.0	14.0	8.00	0.15	0.15	0.07
0.9	15.0	20.0	12.0	0.16	0.20	0.13
9.0	22.0	22.0	18.0	0.21	0.21	0.18
90.0	1.00	21.0	28.0	-0.23	0.21	0.25
900.0	16.0	15.0	20.0	0.17	0.16	0.20
9000.0	21.0	7.00	17.0	0.21	0.05	0.17

viii: Average rate of reproduction and assigned critical values of brown *Hydra* after 7 days of exposure to various concentrations of naproxen. The average rate of reproduction (mean k) is found for each concentration. The difference (d) from the control is then calculated by subtracting each mean k value by the mean k value found for the negative control. The d values collected for each concentration are then divided by the standard error (SE) calculated for each concentration tested (d/SE ratios). Next, a comparison of the d/SE ratios to critical values occurs by ordering the d/SE ratios (from smallest to largest) against a set of critical values (1.79, 1.96, 2.14 …). Any ordered d/SE ratio that exceeds their compared critical value is statistically significant. The LOEC is the lowest concentration that is statistically different from controls ($p \leq 0.05$) and the NOEC is the next lowest test concentration.

Concentration (µg/L)	**Mean K**	**d**	**d/SE**	**Critical Value**
0.0	0.12	0.00	0.00	0.00
0.9	0.16	-0.04	-0.02	2.14
9.0	0.20	-0.08	-0.06	2.50
90.0	0.07	0.05	0.00	1.96
900.0	0.17	-0.05	-0.04	2.32
9000.0	0.14	-0.02	0.00	1.79
*=Significantly different from control at $p \leq 0.05$				

ix: Average number of green *Hydra* and rates of reproduction (k) after 7 days of exposure to various concentrations of diclofenac. Rate of reproduction (k) was calculated by using the equation: $k = [\log_e(ny) - \log_e(nx)] / [ty - tx]$ where nx = number of hydra on first day and tx & ny = the number after y-x days (ty).

Concentration (µg/L)	**Number of *Hydra* Present**			**K Values**		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
0.0	24.0	23.0	22.0	0.22	0.22	0.21
0.9	25.0	24.0	28.0	0.23	0.22	0.25
9.0	38.0	35.0	24.0	0.29	0.28	0.22
90.0	23.0	28.0	33.0	0.22	0.25	0.27
900.0	23.0	33.0	19.0	0.22	0.27	0.19
9000.0	0.00	1.00	0.00	-∞	-0.23	-∞

x: Average rate of reproduction and assigned critical values of green *Hydra* after 7 days of exposure to various concentrations of diclofenac. The average rate of reproduction (mean k) is found for each concentration. The difference (d) from the control is then calculated by subtracting each mean k value by the mean k value found for the negative control. The d values collected for each concentration are then divided by the standard error (SE) calculated for each concentration tested (d/SE ratios). Next, a comparison of the d/SE ratios to critical values occurs by ordering the d/SE ratios (from smallest to largest) against a set of critical values (1.79, 1.96, 2.14 …). Any ordered d/SE ratio that exceeds their compared critical value is statistically significant. The LOEC is the lowest concentration that is statistically different from controls ($p \leq 0.05$) and the NOEC is the next lowest test concentration.

Concentration (µg/L)	**Mean K**	**d**	**d/SE**	**Critical Value**
0.0	0.22	0.00	0.00	0.00
0.9	0.23	-0.02	-0.01	2.32
9.0	0.26	-0.05	-0.01	2.14
90.0	0.24	-0.03	0.00	1.96
900.0	0.23	0.00	0.00	1.79
9000.0	-∞	+∞	+∞*	2.50*
*=Significantly different from control at $p \leq 0.05$ Lowest Observed Effect Concentration (LOEC) = > 9,000 µg/L				

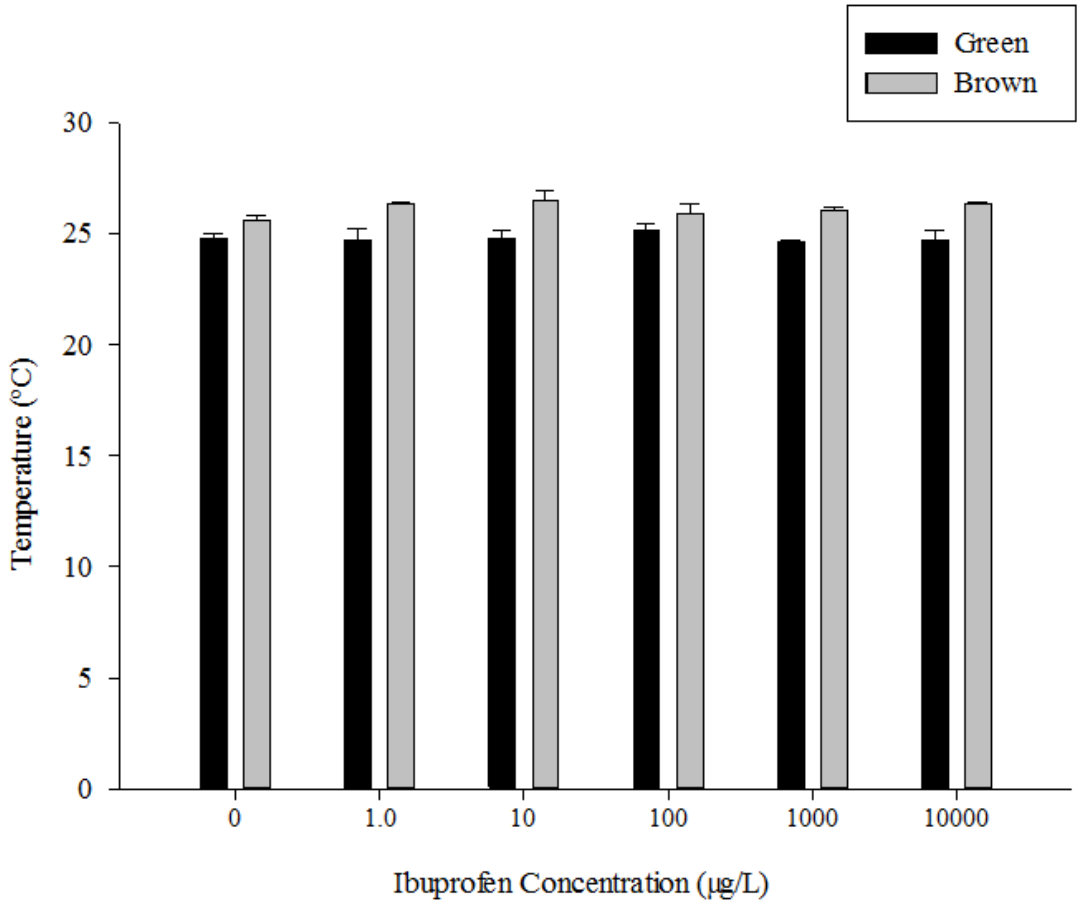
xi: Average number of brown *Hydra* and rates of reproduction (k) after 7 days of exposure to various concentrations of diclofenac. Rate of reproduction (k) was calculated by using the equation: $k = [\log_e(ny) - \log_e(nx)] / [ty - tx]$ where nx = number of hydra on first day and tx & ny = the number after y-x days (ty).

Concentration (µg/L)	**Number of *Hydra* Present**			**K Values**		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
0.0	19.0	6.00	19.0	0.19	0.03	0.19
0.9	7.00	10.0	13.0	0.05	0.10	0.14
9.0	21.0	17.0	14.0	0.21	0.17	0.15
90.0	19.0	24.0	16.0	0.19	0.22	0.17
900.0	13.0	4.00	12.0	0.14	-0.03	0.13
9000.0	0.00	0.00	0.00	-∞	-∞	-∞

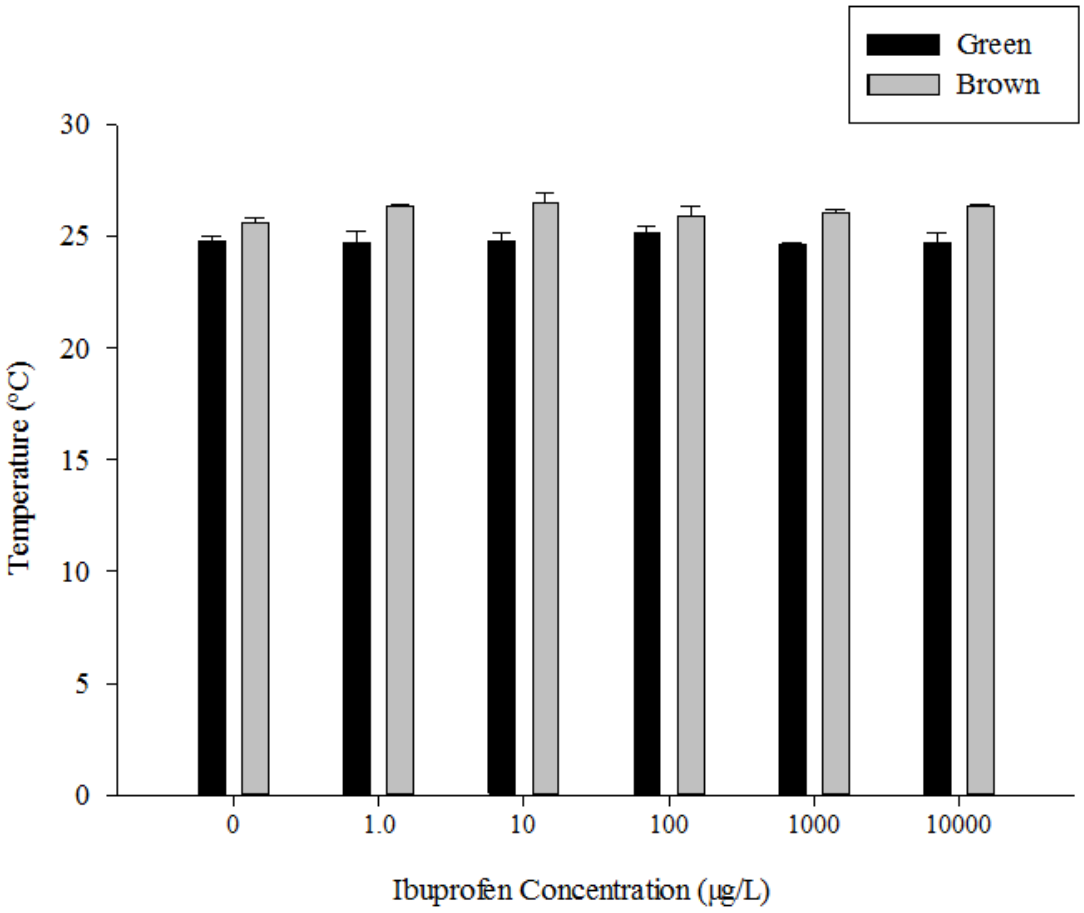
xii: **Average rate of reproduction and assigned critical values of brown *Hydra* after 7 days of exposure to various concentrations of diclofenac.** The average rate of reproduction (mean k) is found for each concentration. The difference (d) from the control is then calculated by subtracting each mean k value by the mean k value found for the negative control. The d values collected for each concentration are then divided by the standard error (SE) calculated for each concentration tested (d/SE ratios). Next, a comparison of the d/SE ratios to critical values occurs by ordering the d/SE ratios (from smallest to largest) against a set of critical values (1.79, 1.96, 2.14 …). Any ordered d/SE ratio that exceeds their compared critical value is statistically significant. The LOEC is the lowest concentration that is statistically different from controls ($p \leq 0.05$) and the NOEC is the next lowest test concentration.

Concentration (µg/L)	**Mean K**	**d**	**d/SE**	**Critical Value**
0.0	0.14	0.00	0.00	0.00
0.9	0.09	0.04	0.02	2.14
9.0	0.18	-0.04	-0.02	1.79
90.0	0.19	-0.06	-0.03	2.32
900.0	0.08	0.06	0.02	1.96
9000.0	-∞	+∞	+∞*	2.50*
*=Significantly different from control at $p \leq 0.05$ Lowest Observed Effect Concentration (LOEC) = > 9,000 µg/L				

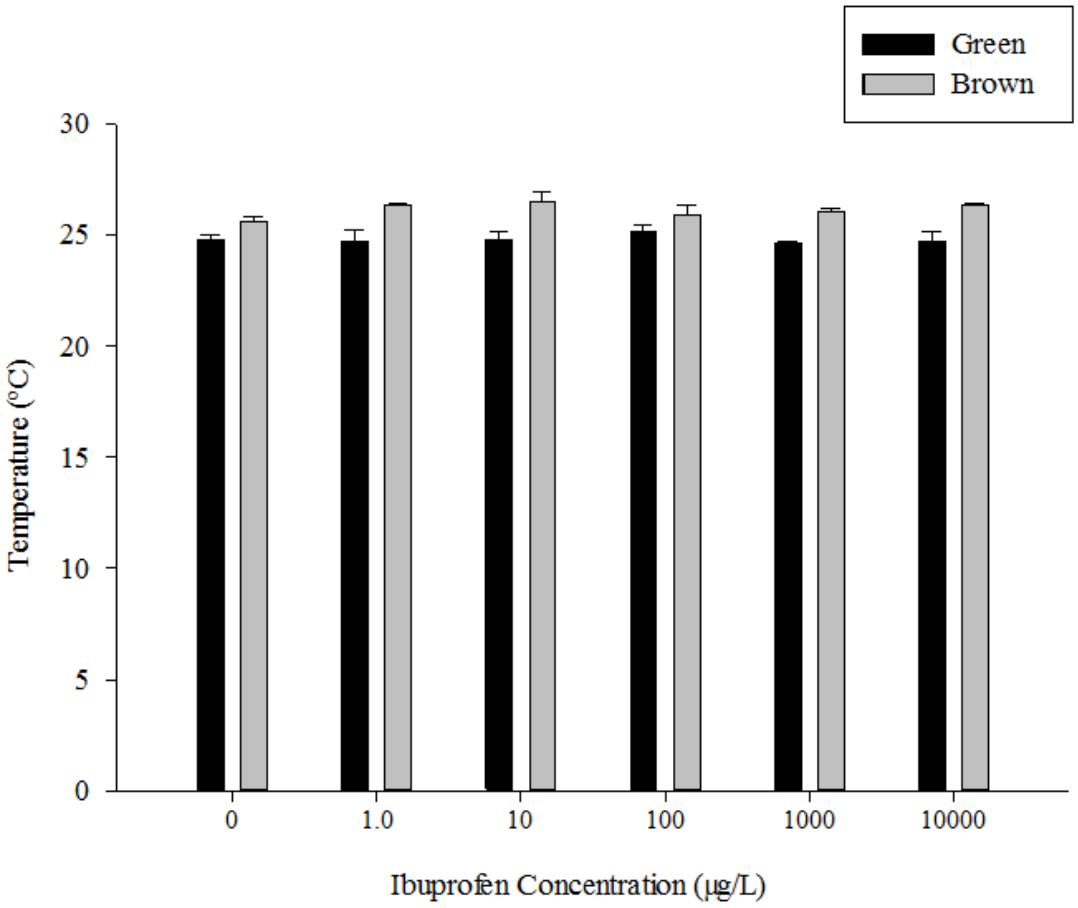
Appendix D: Average temperature (ºC) of green and brown *Hydra* after the 7 day population reproduction tests.



i: Average temperature (ºC) of green and brown *Hydra* after the 7 day population reproduction tests with ibuprofen. The solid bars represent the average value found for 3 wells per concentration and the error bars represent standard error.



ii: Average temperature (°C) of green and brown *Hydra* after the 7 day population reproduction tests with naproxen. The solid bars represent the average value found for 3 wells per concentration and the error bars represent standard error.



iii: Average temperature (°C) of green and brown *Hydra* after the 7 day population reproduction tests with diclofenac. The solid bars represent the average value found for 3 wells per concentration and the error bars represent standard error.