The Acute Toxicity Of Ground Recycled Automobile Tires On Aquatic Life With Model Species P. Promelas

2008

Trillian Baldassari
University of Central Florida

THE ACUTE TOXICITY OF GROUND RECYCLED AUTOMOBILE TIRES ON AQUATIC LIFE WITH MODEL SPECIES *P. PROMELAS*

by

TRILLIAN BALDASSARI, E.I., E.P.I.
B.S. University of Central Florida, 2007

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
in the Department of Civil and Environmental Engineering
in the College of Engineering and Computer Science
at the University of Central Florida
Orlando, Florida

Summer Term
2008

ABSTRACT

Used tires have the potential for becoming popular in pollution control media used in stormwater applications including pervious pavement sub bases, green roof growth media, and upflow filters. Using tire crumb to decrease nutrients can minimize impacts on ecology while reducing the human footprint left by used tires. However, if tire crumb is not examined for toxicity, the ecological balance could unknowingly be disrupted.

This research tested the acute toxicity of tire crumb in aquatic systems by finding the Lethal Concentration for 50% kill (LC50). Using an extreme tire crumb load, *P. promelas* (fathead minnow) were exposed to leachates created with tire crumb and several different types of water including distilled water, tap water, and detention pond water. For distilled and tap water, the addition of tire crumb increased the survival of *P. promelas*. For detention pond water, the addition of tire crumb decreased the survival of *P. promelas*, though only enough to find an LC50 for detention pond water influenced immediately by stormwater runoff. An LC50 was found when 100 percent tire crumb filtrate is prepared with 25 grams of tire crumb per liter of detention pond water collected directly after a storm.

The LC50 found is resultant of a tire crumb load significantly higher than what can be expected in the environment. Based on this research, tire crumb is considered non-threatening to aquatic fish and safe to use with detention pond water.

This thesis is dedicated to three of the greatest women I know

my Grandmother, my Mother & my Sister

ACKNOWLEDGMENTS

Thanks to Dr. Cooper for acting as my major advisor whose guidance and advice were invaluable. Many thanks to Dr. Wanielista for his support, encouragement, knowledge, and great insight. Thanks also to Dr. Chopra for his help and his patience. Special thanks to my water carriers whom without I would have no thesis: David, Patrick, Matt & Brian. Thank you my dear kitty cats, Jaks and Butter, for the happy dance.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS

A. salina	*Artemia salina* (Brine Shrimp)
D. magna	*Daphnia magna* (Water Fleas)
DO	Dissolved oxygen
D. rerio	*Danio rerio* (Zebrafish)
EPA	Environmental Protection Agency
LC50	Lethal Concentration 50
NH_3	Ammonia
NO_2	Nitrite
NO_3	Nitrate as nitrogen
NPDES	National Pollutant Discharge Elimination System
OP	Ortho phosphorus
PAH	Polyaromatic Hydrocarbons
P. promelas	*Pimephaes promelas* (Fathead Minnow)
R. subcapitata	*Raphidocelis subcapitata* (Freshwater Green Alga)
TP	Total phosphorus
UV	Ultraviolet
X. laevis	*Xenopus laevis* (African Clawed Frog)

CHAPTER ONE: INTRODUCTION AND PROBLEM DEFINITION

Introduction

To compensate for the growing number of used automobile tires discarded each year, innovative ways of recycling tires are being implemented. Many of the existing applications, including rubber asphalt and erosion control, expose sensitive environments to tire dust and debris, which could disrupt plant and animal life and change the ecological balance.

Used tires have also been utilized as pollution control media in stormwater applications including pervious pavement sub bases, green roof growth media, and upflow filters. Wanielista et al. (2008) found the use of recycled ground tires can significantly decrease the concentration of ortho phosphorus and total phosphorus in water flowing through the media. A decrease in nitrate, nitrite, and NH_3 was also observed. Using tire crumb to decrease limiting nutrients can minimize impacts on ecology while reducing the human footprint left by used tires.

Objective

The focus of this research is to test the acute toxicity of ground used automobile tires, commonly referred to as tire crumb, in aquatic systems. A Lethal Concentration for 50% kill (LC50) is used. For the purpose of toxicity testing, an extreme tire crumb load will be analyzed. This allows the determination of acute toxicity that is not ecosystem specific and that can be applied to different situations if the tire crumb loading is known.

Limitations

Toxicity testing is one method of assessing the potential ecological impact of tire crumb. A Lethal Concentration for 50% kill (LC50) measures the relative acute toxicity of a substance introduced to a natural environment over a short period (minutes to days) of time. Though considered an effective and acceptable measure of acute toxicity, there is one major limitation as applied here, namely the lack of insight into the long-term effect of tire crumb on an ecosystem.

Another limitation includes the amount of tire crumb and method in which tire crumb is exposed to test organisms. It is difficult to determine how much crumb rubber is deposited in the environment from its various uses over time. In order to find the potential toxicity of crumb rubber in a natural body of water, it would be necessary to estimate the loading.

CHAPTER TWO: BACKGROUND

Waste Tires

Finding new applications for used automobile tires is increasingly important with the amount of tires generated in the hundreds of millions. Crumb rubber is a term used to describe recycled rubber from the automotive industry that has been processed into coffee grain size particles. Used tires are typically ground into two different classes, 10 mesh and smaller and larger than 10 mesh, termed ground and coarse rubber respectively. With two classes of particle sizes in the tire crumb market, the potential applications are innumerable. However, the single largest market for ground rubber is rubberized asphalt, consuming an estimated 12 million tires annually. Florida is currently a leader in asphalt rubber utilization (“Ground Rubber Applications,” 2007).

Another potential use, though small in comparison to rubberized asphalt, is the use of crumb rubber as a filter in wastewater applications. Studies have shown that tire crumb can be used as an effective filter medium achieving similar results compared to using a sand/anthracite filter to remove turbidity and suspended solids. It was also indicated that the head loss associated with running water through tire crumb as apposed to the standard sand/anthracite medium is significantly less. Using crumb rubber for wastewater filtration was also shown to be additionally beneficial by reducing capital and operational costs (Xie, 2007).

Crumb rubber was also tested to see how it would fare in the filtration of ballast water. It performed favorably in the removal of turbidity, particulates, phytoplankton, and zooplankton. Greater removal efficiencies were experienced with smaller particle sizes and lower filtration rates. The greatest efficiencies were experienced with the smallest particle sizes, ranging from 0.5 to 1.2 mm, though sizes up to 4 mm were still found to be effective. The study also found that the use of crumb rubber filters was additionally beneficial, requiring less backwash water than other filter media (Tang et al., 2006).

Tests were conducted on the feasibility of using tire crumb to replace pea gravel on putting green plots. In this study, traditional pea gravel was replaced with equally sized tire crumb in simulated putting green columns. It was observed that tire crumb helped to significantly reduce nitrate leaching. This led to the conclusion that using tire crumb as a distinct sub-layer beneath sand-based root zones can help reduce nitrogen contamination of water bodies by preventing nitrogen from migrating unrestricted. However, tire crumb cannot eliminate nitrogen contamination of water bodies altogether. This study also made a very important observation for putting green applications in that tire crumb did not adversely affect the establishment, density, quality or color of the grass.

Research has shown that there is also potential to use ground, discarded tires to remove organic and inorganic contaminants from wastewater. One of the components of automobile tires is carbon black, which functions similar to activated carbon. Tire crumb generally contains 27 to 33 percent carbon black making it a good sorbent that can effectively remove dissolved organic substances (Gunasekara et al., 2000). It was found that ground, discarded tire rubber has great potential for adsorbing organic compounds such as naphthalene and toluene, which are present in many contaminated sites and are considered threats to human health.

Another potential application utilizes recycled tires in erosion control. In California, several discarded tire erosion control applications have been designed and implemented. Tires have been used in applications such as reinforcing unstable highway shoulders or protecting channel slopes (Jan et al., 1998). In this application, whole tires are typically used as opposed to ground tires. To form a stable frame, the tires are generally banded together and then either partially or completely buried.

Toxicity of Waste Tires

Tire crumb is composed of 85 percent carbon, 10 to 15 percent ferric material, and 0.9 to 1.25 percent sulfur. Though most steel, fiber, and other contaminants are removed from the used tires prior to recycling, they may be present in tire crumb in trace amounts (Global Tire Recycling, 1998). When tire crumb is allowed to equilibrate with water, there is potential for known toxic chemicals such as zinc, copper, chromium, and lead to leach into the water. Whether the levels of known toxins will be high enough to cause a toxic effect is unknown.

One study tested tires from different applications and life stages. These included both new and road-worn tires as well as tires previously used as a floating breakwater to protect marine structures from wind and waves. Whole tires were immersed in 300 L of water and samples were taken at 5, 10, 20 and 40 days to study their static lethality. It was found that leachate created from the used tires was more lethal to rainbow trout than leachate created from new tires. It was also found that the leachate was toxic to rainbow trout after the tires were removed from the water indicating that the toxicants degraded very slowly. Neither of these leachates was found to be toxic to fathead minnows. In this study, it was reported that leachate obtained from floating breakwater tires was not toxic to any species (Day et al., 1993). Due to the nature of breakwater applications, the lack of toxicity could be indicative of toxic substances leaching from the breakwater tires prior to the start of the experiment.

Another way tires can impact the environment is through the accumulation of tire dust, often called tire debris, which is formed by the degradation of tire tread while driving. Tire debris has a very large surface area and a very complex chemical composition. Some of its components are water-soluble and can be easily leached. A certain quantity of zinc is released by tire debris when it rains, which is particularly soluble at low pH values and may be carried in stormwater runoff. Many reports have identified zinc as the major toxicant in aquatic environments when automobile tires are utilized (Guiltier et al., 2005).

One study researched the effect of tire debris on living organisms in a laboratory setting under controlled conditions as well as under similar conditions to what would be found in the environment. The particles used in the laboratory had similar shape and dimension to those collected in ambient air. The leachates were tested on *Raphidocelis subcapitata, Daphnia magna,* and *Xenopus laevis* developing embryos (Guiltier et al., 2005). In this study, it was found that pH, dimension of the particles and particle aggregation all influenced the quality of the leachate. It is suggested that the toxicity is not only related to the amount of zinc that is leached out, but also the amount of organic chemical compounds (Guiltier et al., 2005).

To determine the particle size distribution that can be expected to be found in the environment as a result of normal tire wear, wear tests were conducted on new vehicle tires and the resultant particles were analyzed. It was found that particles were produced in the range of 10 to 80 μm (Guiltier et al., 2005). Then, leachate was produced using the lab created tire particles at a pH of 3, which is the lowest pH value of acid rain caused by anthropogenic activities (Guiltier et al., 2005). In order to obtain a variety of leachates, 50 and 100 grams of tire debris were put in 1 L glass bottles containing water at pH ranging from 3 to 7. The bottles were put in a mechanical shaker where they were shaken for 24 hours at a speed of 50 rpm. In addition, different concentrations of the leachate at a pH of 3 were prepared (Guiltier et al., 2005).

This allowed for the quantification of zinc leached from tire debris at different pHs. The quantity of zinc was measured in the undiluted samples. Each leachate was tested for toxicity on living organisms at concentrations of 1, 10, 50, and 100 percent (Guiltier et al., 2005). As would be expected, the concentration of zinc at lower pH was found to be higher than that at higher pH.

Both the quantity of zinc and the toxicity were found to be higher in solutions made with 50 grams of tire debris per liter of water than with 100 grams per liter. It was noted that there is a non-linear relationship between the quantity of tire debris and the concentration of zinc in the leachate. It was found that a higher concentration of tire debris tends to form an aggregate that exposes a lower surface area and inhibits leaching (Guiltier et al., 2005).

Leachates created using tire wear were tested on *Daphnia magna*, commonly called water fleas, to determine the potential toxicity. In order to test if toxic compounds leach from tires, rubber was allowed to equilibrate with dilution water for 72 hours at 44°C, which was considered the worst case scenario as leaching is known to occur more rapidly at higher temperatures and would represent the temperature of a road on a hot summer day. In the filtered samples, it was determined that most of the toxicity resulted from non-polar organic compounds. It was also found that the toxicity of all leachates increased with exposure time (Wik et al., 2006).

Other studies using *D. magna* as test organisms have found that leachate derived from whole tires, about 33 grams of rubber per liter, was non lethal while tire pieces leached at 200 grams per liter were found to have a 72 hour LC50 of 12.5%, or 25 grams per liter. One of the reasons for this difference in toxicity between the different forms of rubber is that metals are often leached out of cut or shredded tires, but not out of whole tires (Wik et al., 2006).

In the same study, it was found that when the water containing the daphnids was placed underneath a UV light for 2 hours, several of the leachates showed a significant increase in toxicity (Wik et al., 2006). It is suggested that the photo-enhanced toxicity of the unfiltered samples is caused by PAHs, which are known to be photo toxic. Though, it was found that the ratios of photo-enhanced toxicity did not vary significantly from the controls.

There is high variability between the toxicity found in this study and in others, which could be due to differing methods and materials. Variability has been found to be resultant of the salinity of the test water, the age of the tested tires, as well as the different rubber formula used by different tire manufacturers. When reviewing the content of 29 different brands and sizes of tires, significant variation was found in the amount of cobalt, aluminum, and lead (Wik et al., 2006).

The methods of which leachate is made can vary the potency greatly. The pH used to make the leachate can determine the concentration of metals and PAHs that leaches out of the tires. In terms of making leachate, it was also pointed out that leachate prepared at lower loading rates results in higher toxicity. This puts even more importance on the procedure for preparing the leachate because it is apparent that allowing different amounts of tire pieces to equilibrate with water will lead to different leachate concentrations (Wik et al., 2006). The zinc concentration for the different tire leachates ranged from 110 to 590 µg/l (Wik et al., 2006).

Acute Chemical Sensitivity of Freshwater Organisms

Martins, et al (2004), found that acute toxicity assays with *D. magna* and *D. rerio* can give important and relevant information concerning the possible human oral chronic intoxication and could be used as an initial screening of toxicity.

In a study, it was found that some substances were markedly more toxic to one of the test species than the other. For the majority of such instances, substances are more toxic to *D. magna* than *D. rerio*. Very rarely was it found that a substance was more toxic to *D. rerio* than *D. magna*. For the chemicals that are more toxic to *D. magna*, crustaceans could be used to predict acute toxicity to fish. In the instance where *D. magna* is used to predict toxicity in fish, it is beneficial that tests involving *D. magna* have a 48-hour exposure time compared to a 96-hour exposure time in *D. rerio*. This could be extremely important when faster answers are needed when facing potential environmental contamination (Martins et al., 2004).

Chronic Chemical Sensitivity of Freshwater Organisms

Currently, when studying the toxicity of a pollutant, the main focus is on the mortality of the test specimens. However, environmental pollutants can negatively impact aquatic ecosystems at much lower concentrations than necessary to kill a specimen. Many pollutants can have an impact on the physiology of animals, which may impair their ability to survive in the long term. Pollutants can alter basic life functional ability, such as impairing the ability to either hunt prey or hide from predatory animals.

Traditionally, regulatory guidelines are based on lethality tests, such as the 96-hour LC50. These tests fail to examine what could happen to an aquatic system if lower concentrations of the pollutant are allowed to enter the system. Even if the test specimens are not killed by the contaminant, their normal behavior may be altered, preventing them from being able to function in an ecological context (Scott et al., 2004).

There is much known about the physiology of fish and these normal behaviors can be observed and quantified in controlled environments. For this reason, fish behavioral indicators may be used to monitor levels of environmental contamination. Toxicants may disrupt or initiate specific physiological sequences, causing inappropriate behavioral responses, which could result in detrimental behavioral alterations having severe implications for survival (Scott et al., 2004).

The majority of research has discussed direct behavioral responses of fish. Only recently has research turned to the impact on the more complex behaviors, such as social hierarchies and reproduction. These less obvious behavioral alterations can pose a much larger threat to the overall health of the ecosystem. Behavioral indicators are likely to be ideal for assessing sub-lethal impacts of pollutants (Scott et al., 2004).

Predatory-prey interactions can be altered by sub-lethal doses of a pollutant by altering the ability of prey to avoid a predator by altering their response to a potential predation risk, such as altering the schooling abilities of a group. This increase in the likelihood of a predator catching a prey increases the contamination level as you move up the food chain (Scott et al., 2004). This could essentially disrupt an entire aquatic ecosystem.

Pollutants can also alter reproduction procedures. Spawning of fish involves many interrelated steps. For instance, if two connected life functions were to include the defense of spawning site and nest building and one of these steps were to either fail to occur in a timely manner or fail to occur all together, the following steps, such as courtship and spawning, could also fail to occur, altering the reproductive success of a species. Though most studies have shown that nest building, spawning, and courtship behaviors are frequently interrupted by pollutants such as trace metals and organic pollutants, behaviors such as spawning site selection and natal homing could also be altered by pollutants (Scott et al., 2004).

Scott et al. (2004) has shown that environmental pollutants can also affect the social interactions associated with territory and dominance of fish. At different concentrations, fish may either show greater or lesser levels of agonistic interactions thus disrupting the social hierarchy of fish populations. The hierarchy of fish populations is established to allow the top fish optimal feeding and larger growth rate. This hierarchy has been shown to promote population stability.

Choice of Species

Model species are chosen so that information obtained from examining them can be used to generalize about other groups of species. For this reason, the species chosen to be model species are typically non-specialized. The behaviors necessary for fish survival are dependent on many physiological systems including sensory, hormonal, neurological and metabolic. The impact of pollutants on each of the systems should be considered.

It is important to test a variety of suitably representative organisms to determine the toxicity of chemicals to understand their potential effects on both humans and the health of the environment (Teather et al., 2006).

A variety of fish is used to test the toxicity of chemicals, including fathead minnows and rainbow trout. However, the sensitivity each individual specimen varies according to the type and concentration of toxicant. Comparing the sensitivity of each specimen to different toxicants is expected to be useful for predicting the responses of different species to untested chemicals. Some species may react to chemicals in a way that is specific to the particular species or some may react to a different degree than others. For instance, certain species absorb, metabolize, or excrete chemicals more than others. Generalizing the impact a pollutant may have on an ecosystem based on the results of one species may have negative implications.

According to Teather and Parrott (2006), it remains unclear whether the difference in species sensitivity remains consistent across an array of chemicals. After reviewing all studies that tested the LC50 of a chemical, it was found that the three most common test specimens were fathead minnows, rainbow trout, and bluegill sunfish. After analyzing all of the previous studies that contained both fathead minnows and rainbow trout, Teather and Parrott (2006) determined that fathead minnows are only about forty-two percent as sensitive as rainbow trout, making the rainbow trout quite a bit more sensitive to most chemicals than the fathead minnow. If studies existed that contained rainbow trout and bluegill sunfish, guppies or goldfish, the results were also analyzed. It was observed that rainbow trout were significantly more sensitive to the acute effects of the chemicals tested.

Once it was determined that rainbow trout do, overall, exhibit increased sensitivity to chemicals, the studies were analyzed to determine if the rainbow trout and fathead minnow exhibit differential sensitivity to different classes of chemicals. Teather and Parrott (2006) found that trout were almost 10 times as sensitive to metals, fathead minnows were significantly more sensitive to hydrocarbons, and the two species showed equal sensitivity to CH-chains and phthalates. Though the information obtained from the various studies shows the rainbow trout is dramatically more sensitive to metals, there is very little information concerning the age of the test specimens. Generally, juveniles exhibit greater sensitivity to toxicants.

An interesting example of the varying sensitivity of different species is the relative toxicity of waste tires to rainbow trout compared to *D. magna*. Previous literature found that leachate obtained by leaving scrap tire in water for 60 days caused 100 percent mortality to rainbow trout within 24 hours. However, this same leachate was found to be nonlethal to *D. magna* (Guiltier et al., 2005).

Bioassays and Toxicity Testing

Toxicity tests are vital to examining questionable compounds and their potential to harm life in aquatic systems. For this reason, the Environmental Protection Agency recognizes several different exposure assessment models, including the LC50, to examine aquatic sensitivity and the impact a pollutant may have on a natural body of water. Exposure assessment models can help reduce the reliance on uncertainty factors in ecological risk assessment ("LC50," 2007).

A toxicity test exposes carefully chosen indicator organisms to different concentrations of a questionable pollutant to observe the pollutants effect on the organisms ("Bioassay," 2007). A toxicity test can measure either acute or chronic effects. Acute toxicity tests measure how well an organism can survive and chronic tests measure sub-lethal effects, including the effect on an organism's ability to grow and reproduce ("Toxicity Testing," 2007). Both acute and chronic toxicity tests are important to protect the overall health of an ecosystem.

Acute tests can run for twenty-four, forty-eight, or ninety-six hours. LC50 is an acute toxicity test that specifically measures the concentration of a pollutant in an aquatic system that is lethal to fifty percent of the test animals in a given amount of time, generally 96 hours.

Short-term chronic tests can run from seven to nine days, depending on the test organism. The most common test organisms for both acute and chronic testing include water fleas, fathead minnows, bannerfin shiners, mysid shrimp, and tidewater silversides ("Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms," 2006).

As with many tests, the number and concentration of dilutions and replicates directly affects the quality of the data. To obtain quality data, the maximum feasible number of dilutions, test organisms, and test replicates are necessary. However, with a bioassay, there are far more factors involved in quality assurance. For instance, the initial condition of the test organisms can greatly impact the test results. For best results, it is important to be consistent with the groups tested, but also to have test samples with organisms of various ages and sizes ("Toxicity Testing," 2006).

In addition to maintaining healthy organisms at the start of a bioassay, it is important to maintain healthy organisms throughout the duration. The test organisms must be handled very carefully at the beginning of the test to not injure them before exposing them to the test concentrations. The organisms must be handled carefully during the test while the test chamber is cleaned and chemical adjustments are made if necessary. They must also be fed regularly ("Toxicity Testing," 2007).

Though a high level of quality may be maintained by the source of test organisms, there are many different areas in which toxicity test variability may be introduced and minimized with proper attention. For instance, shipping and handling samples in a consistent manner can reduce response variations. Variability can also be introduced if samples need to be manipulated prior to testing. However, consistency in adjustment methods will help to reduce variability ("Toxicity Testing," 2006).

Toxicity Test Guidelines

The EPA developed a manual for measuring acute toxicity of effluents and receiving waters to freshwater organisms. Standard Methods also developed guidelines for the testing of toxicity on freshwater species. Section 8910 Fish and Section 8020 Quality Assurance and Quality Control in Laboratory Toxicity Tests were both referenced (American Public Health Association, 2005). The main difference in the two guidelines is the EPA manual is meant for use in the NPDES Permits Program and is significantly more detailed while Standard Methods provides more general toxicity test guidelines. It should be noted that much of the Standard Methods procedure comes from the EPA's Methods of Measuring the Acute Toxicity of Effluents and Receiving Waters of Freshwater and Marine Organisms.

After detailed review of both guidelines, it is apparent that certain components be included in any toxicity test. Of all the guidelines, two components appear to be the most substantial. The first component of interest is the age and species of the test specimens. It is important that the test species be closely related to a species found in the relevant aquatic systems. It is also important that the species be easily cultured in the laboratory and sensitive to a variety of pollutants. In this instance, fathead minnows have been selected. The age of the specimens is also important because organisms are more sensitive to toxicants in the early stages of life.

The second component of interest is the overall setup of the experiment. Both guidelines recommend that each test should include a control group and five different concentrations of the toxicant. For each concentration, it is further recommended that a minimum of 20 specimens be tested within a minimum of 2 test chambers made of tempered glass. All testing shall be performed based on these two main components.

Table 1 summarizes the required and recommended test conditions from EPA's Methods for Measuring the Acute Toxicity of Effluents and Receiving Water to Freshwater and Marine Organisms. For a more detailed summary, please refer to Appendix B.

Table 1: Summary of Test Conditions[1]

Summary of Test Conditions and Test Acceptability Criteria for Fathead Minnow, *Pimephales Promelas,* Acute Toxicity Tests with Effluents and Receiving Waters	
Test type	Static non-renewal, static renewal, or flow-through
Test duration	24, 48, or 96 h
Temperature	20°C ± 1°C; or 25°C ± 1°C (recommended). Test temperatures must not deviate by more than 3°C during the test (required)
Light quality	Ambient laboratory illumination (recommended)
Light intensity	10-20 µE/m2/s (50-100 ft-c) (ambient laboratory levels) (recommended)
Photoperiod	16 h light, 8 h darkness (recommended)
Test chamber size	250 mL (recommended minimum)
Test solution volume	200 mL (recommended minimum)
Renewal of test solutions	After 48 hours (required minimum)
Age of test organisms	1-14 days; less than or equal to 24-h range in age (required)
No. organisms per test chamber	10 for effluent and receiving water tests (required minimum)
No. replicate chambers per concentrations	2 for effluent tests (required minimum) 4 for receiving water tests (required minimum)
Feeding regime	*Artemia* nauplii are made available while holding prior to the test
Test chamber cleaning	Cleaning not required
Test solution aeration	None, unless DO concentration falls below 4.0 mg/L
Dilution water	Moderately hard synthetic water, receiving water, ground water, or synthetic water, modified to reflect receiving water hardness (available options)
Test concentrations	Effluents: 5 and a control (required minimum)
Dilution series	Effluents: ≥0.5 dilution series (recommended)
Endpoint	Mortality (required)
Sampling and sampling holding requirements	Receiving Waters: Grab or composite sample first used within 36 h of completion of the sampling period (recommended)
Sample volume required	2 L for effluents and receiving waters (recommended)
Test acceptability criterion	90% or greater survival in controls (required)

[1] Adapted from US EPA (2002)

CHAPTER THREE: APPROACH AND EXPERIMENTAL DESIGN

Approach

One of the problems faced in determining the toxicity of crumb rubber is finding the best method for exposing the test specimens, in this case fathead minnows, to the rubber. One method is to expose fathead minnows to tire crumb by allowing the tire crumb to equilibrate with water for 72 hours with the aid of an air pump assuming any toxic substances should leach from the tire crumb within this period of time. At the end of 72 hours, the tire crumb will be removed from the water creating a filtrate that can then be easily tested for toxicity at varying concentrations.

One limitation in this approach is the length of time chosen for equilibration. It is necessary that the equilibration time be sufficient to allow toxic substances to leach out. However, it is also important that the equilibration period not be unnecessarily long such that testing cannot be completed within a reasonable amount of time. An equilibration time of 90 days, for instance, would require years for testing multiple concentrations and water types. Researchers have found 72 hours a sufficient amount of time to allow a substance to equilibrate with water (Wik et al., 2006). To examine the difference in equilibration durations, leachates were allowed to equilibrate for 72 hours and 30 days and their composition analyzed. In Table 2, shown is the breakdown of the different filtrates. It should be noted that that allowing the tire crumb to equilibrate for 30 days does not significantly change the composition of the filtrate.

Table 2: Comparison of Equilibration Times for Tire Crumb Filtrate

	Tire Crumb Filtrate Equilibration Time: 72 hours	Tire Crumb Filtrate Equilibration Time: 30 days
Date	10/22/07	10/22/07
pH s.u.	6.57	6.60
Alkalinity mg/l	14.0	18.0
Copper µg/l	17	19
Lead µg/l	9	9
Barium µg/l	<2	6
Chromium µg/l	2	<2
Zinc µg/l	2878	2714
Iron µg/l	126	146
NOX-N µg/l	18	35
SRP µg/l	1	6
Mercury µg/l	0.11 U	0.11 U
Silver µg/l	0.77 U	0.77 U
Phenol mg/L	0.025 U	0.16
VOC	x	x

For each of the different experiments, 25 grams of crumb rubber per liter of water was used to make the filtrate. In order to determine the magnitude of loading that can be expected, a typical stormwater application of tire crumb was analyzed (Ryan, 2008). Ryan used an upflow filter using 12 ft^3 of media with 45 percent tire crumb in a detention pond with a permanent pool volume of 12 acre-ft. Based on this application, the whole pond will only be exposed to 0.004 grams of tire crumb per liter of pond water. Though it remains unclear whether the average detention pond will be exposed to more or less than 0.004 g/L tire crumb, 25 g/L tire crumb is still considered an extreme load.

For each experiment, the filtrate was tested using four different concentrations and one control. For quality assurance purposes, the tests should be conducted in triplicate, using one aquarium for each of the different test chambers. As a general rule, fish should be allowed a gallon of water per inch of fish. The approximate size of 6 day-old fathead minnows is a quarter of an inch. With EPA's specification of 10 fish per chamber, 2.5 gallon aquariums are sufficient.

To reduce the margin of error due to stress of the organisms, test organisms should be received at 4 days old and tested when the organisms are 6 days old, allowing 2 days for acclimation to testing conditions. This was noted after the first experiments experienced significant loss in all scenarios when using fish without a two-day acclimation period. When the fish are allowed to acclimate for 48 hours, the weaker specimens expire leaving only the healthy specimens to be tested. Due to the age of the organisms, fresh brine shrimp were hatched and fed every day beginning the day of arrival of the test organisms until the end of the test.

Two different sets of data are desired. The first set of data sought after is the raw toxicity of crumb rubber with as little interference as possible. To achieve this, distilled water and tap water are both used, with and without an aquarium buffer and dechlorinator. The second set of data sought after is the effect of tire crumb on natural pond water. In this experiment, water is to be harvested from the Pegasus Pond on the University of Central Florida campus for two different scenarios. The first scenario is regular pond water and the second scenario is pond water after a rainfall event. In this instance, one day and eleven days after a 2.83-inch rain occurring April 6, 2008. For pond water, the total amount needed was colleted within an hour to reduce variability in the sample.

During the course of the experiment, the dissolved oxygen, the pH, the temperature, and the number of fish alive were be observed and recorded. Testing at the beginning and every 24 hours until the completion of the test should be sufficient to determine if loss of life is resultant of high pH or low dissolved oxygen.

Experimental Design

The tire crumb was allowed to equilibrate with water for 72 hours in 10-gallon aquariums prior to each experiment. An air pump was used to aid in the mixing of the tire crumb. With a capacity of 35 liters, 875 grams of tire crumb is needed for each filtrate aquarium to obtain a concentration of 25 grams per liter. Once the equilibration period finishes, the tire crumb was removed from each aquarium and discarded. Because research has shown that the toxicity of tire crumb may be reduced by exposure to water, tire crumb should not be reused in toxicity testing (Day et al., 1993). It is important that the filtrate aquariums not be in close proximity to the testing aquariums.

It was arranged that 4-day old fathead minnows arrive 48 hours prior to each experiment. Upon arrival, minnows were transferred to a holding chamber in the same water they arrived in. For the next 48 hours, dead and weak minnows are to be culled.

It was also arranged that fresh brine shrimp hatch the day new fish arrive and each day subsequent to their arrival. Young minnows were fed freshly hatched brine shrimp until they are old enough to eat commercial fry food, generally occurring around three weeks of age.

The testing chambers consisted of 2.5-gallon glass aquariums. To accommodate one control group and five different concentrations of filtrate, 18 testing chambers are necessary. The breakdown of the different concentrations is as follows:

Table 3: Filtrate Concentration Breakdown

Percent Tire Crumb Filtrate	Filtrate Volume (L)	Water Volume (L)	Total Volume (L)
100	8	0	8
50	4	4	8
25	2	6	8
12.5	1	7	8
6.25	.5	7.5	8

Filters and air pumps are not required. When the filtrate is ready, it should be transferred to the testing chambers in the required concentrations with the base of the filtrate used as dilution water. For instance, if tap water is used as the base for the tire crumb filtrate, tap water is used as the dilution water. If it is necessary to use multiple aquariums for the preparation of filtrate, equal volume should be pulled from each of the aquariums when the filtrate is harvested for each of the concentrations.

Once the test chambers were prepared, minnows were added one at a time to each of the chambers until each chamber had 10 minnows. This ensures that the weaker specimens are not caught first and all added to the first chamber. Minnows should be handled using a brine shrimp net to avoid damage during transport. Each minnow should be observed upon transport to ensure they are not harmed during the transport process.

The pH, dissolved oxygen, and temperature should be measured and recorded at zero hours and every 24 hours for a total of 96 hours. The minnows should be observed every 24 hours and expired specimens culled. Specimens are considered expired when immobile and fail to respond to a stimulus. In this case, creating a gentle movement in the water near the specimen in question will create enough of a stimulus to determine if the specimen has expired.

At the end of the experiment, all remaining minnows are to be transferred to a 10-gallon aquarium where they should be fed and cared for. Testing chambers should be emptied and cleaned.

CHAPTER FOUR: RESULTS AND DISCUSSION

Distilled Water-Based Filtrates

The following chart illustrates the percent survival of fathead minnows exposed to filtrate prepared using distilled water as a base. For 100 percent tire crumb filtrate, no distilled water is used to dilute the filtrate. However, for 0 percent tire crumb filtrate, minnows are exposed to 100 percent distilled water.

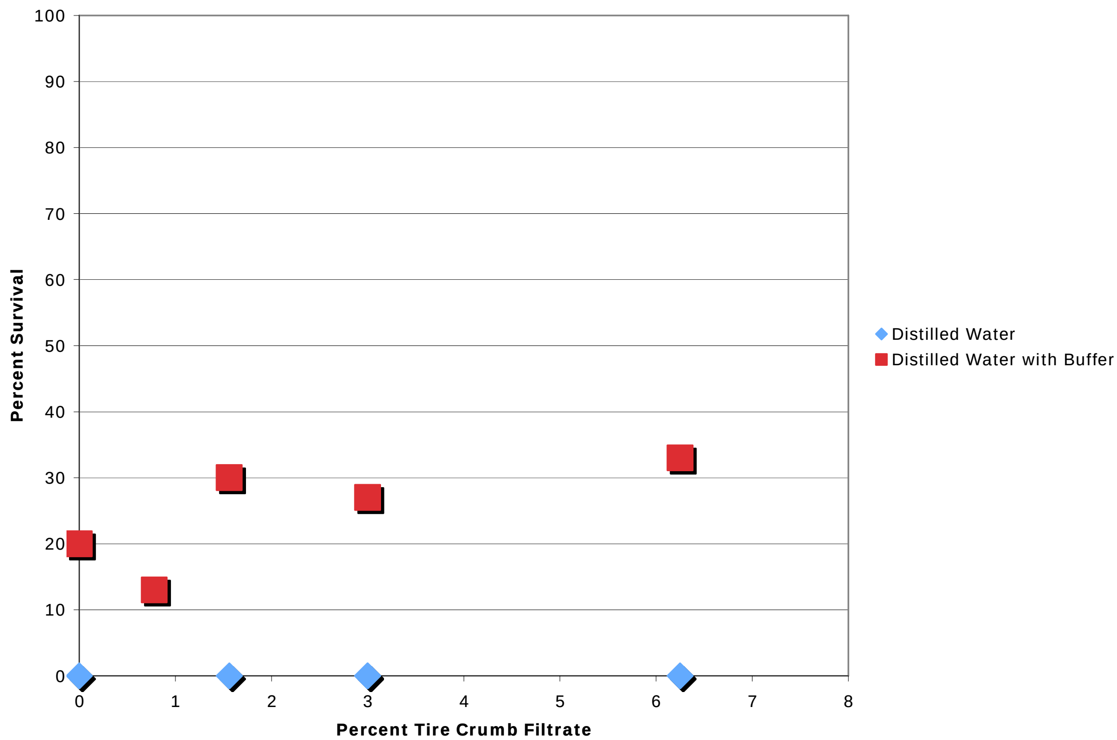

Figure 1: Percent Survival for Distilled Water-Based Filtrates

As shown, all concentrations of tire crumb filtrate prepared using distilled water were 100% lethal to fathead minnows, including the control. This is likely due to the lack of essential minerals and buffering capacity that a natural system would contain.

The second data set illustrates the percent survival when exposed to filtrate prepared using distilled water with a buffer as a base. As shown, the survival rates tend to increase with increasing percent of tire crumb filtrate, though the overall survival is still relatively low.

An LC50 cannot be determined due to the low levels of survival in the control groups.

Tap Water-Based Filtrates

In Figure 2 are illustrated the two data sets for the survival of fathead minnows exposed to filtrate prepared using tap water as a base. The same method of preparing the different dilutions for percent tire crumb filtrate was used with 100 percent tire crumb filtrate consisting of an undiluted sample. For both the tap water with and without buffer, survival rates tend to increase with increasing tire crumb filtrate concentration.

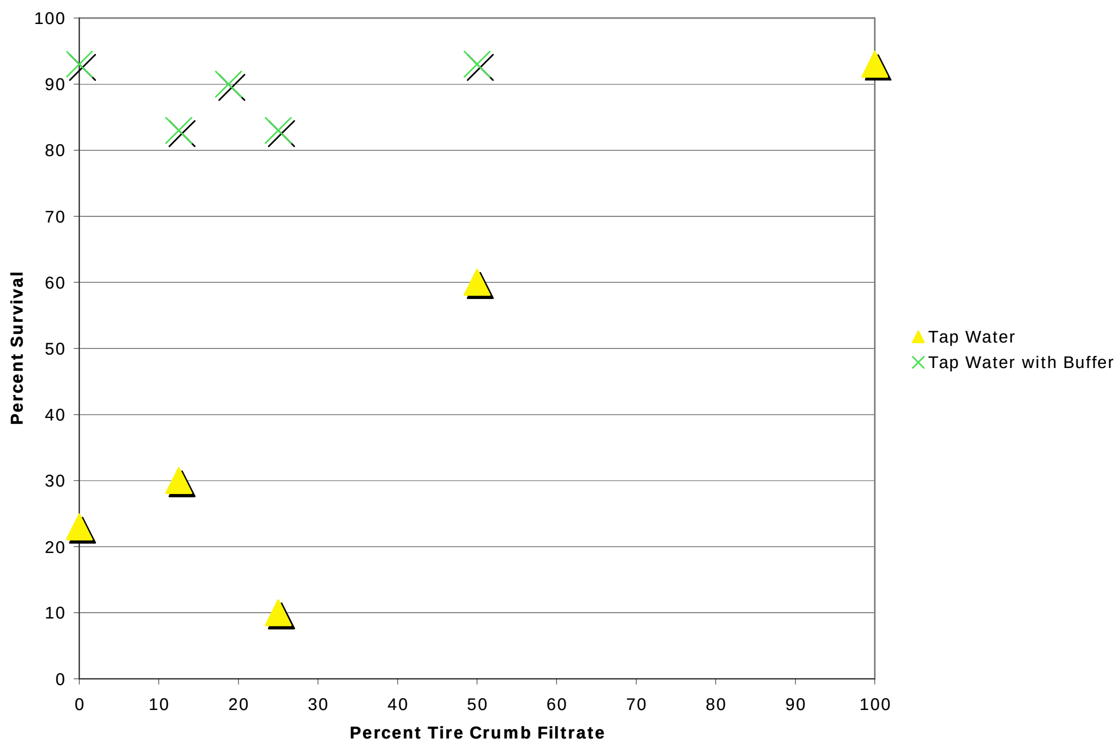

Figure 2: Percent Survival for Tap Water-Based Filtrates

Though the tap water without buffer experienced survival rates near fifty percent, an LC50 cannot be determined due to the low survival rate of the control group.

Pond Water-Based Filtrates

The following chart illustrates the percent survival of fathead minnows exposed to filtrate prepared using pond water as a base. For lower concentrations of tire crumb filtrate, the pond water collected immediately after the storm exhibits higher percent survival than the pond water colleted 11 days after the storm. However, for 100 percent tire crumb filtrate, the pond water immediately after the storm shows a lower percent survival.

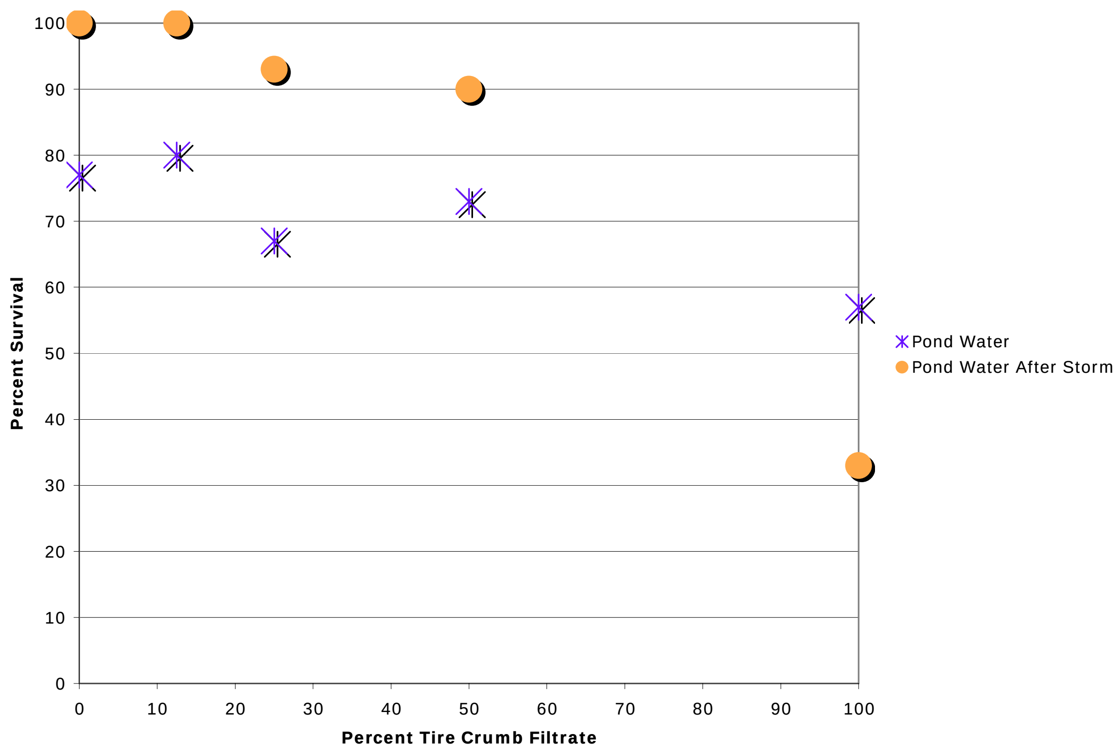

Figure 3: Percent Survival for Pond Water-Based Filtrates

An LC50 for typical pond water was not found because the survival rate was consistently greater than fifty percent. However, an LC50 of 100 percent tire crumb filtrate prepared with 25 grams per liter pond water immediately after a storm was observed.

CHAPTER FIVE: CONCLUSION AND RECOMMENDATIONS

Summary

With a growing number of pond water applications for tire crumb, it has become increasingly important to test the toxicity of tire crumb to aquatic life. Both EPA and Standard Methods provide guidelines for toxicity testing using freshwater organisms, which provided a basis for experimental design.

Because fathead minnows are commonly found in Central Florida ponds, fathead minnows are the selected test species. The fathead minnows are exposed to leachates created with tire crumb and several types of water as a base. The different types of water include distilled water, distilled water with an aquarium buffer, tap water, tap water with an aquarium buffer, detention pond water, and detention pond water immediately affected by stormwater runoff. Exposing specimens to leachate with distilled water and tap water as a base intends to find the toxicity of crumb rubber with as little interference as possible. Exposing specimens to leachate with pond water as a base intends to find the toxicity of crumb rubber in a setting similar to that which the crumb rubber will be applied.

Tire crumb filtrate was prepared by allowing tire crumb to equilibrate with water for 72 hours. The tire crumb was then filtered out. Specimens were exposed to tire crumb filtrate in 2.5-gallon aquariums using three test chambers per concentration. A total of 30 specimens were exposed to each concentration. Every 24 hours during the 96-hour duration, the pH, dissolved oxygen, temperature, and number of live specimens were recorded.

Conclusion

Tire crumb is not found to be toxic when testing with tap water and distilled water. In tap water and distilled water instances, tire crumb filtrate increased the survival of fathead minnows to greater rates than experienced in the control chambers. In the case of distilled and tap water, the tire crumb could either offer a constituent necessary for survival, or adsorb a substance preventing the survival of fathead minnows.

In detention pond water not effected immediately by stormwater runoff, tire crumb is found to lower the survival rate of fathead minnows, though not low enough to determine the LC50 of tire crumb leachate made with 25 grams per liter of pond water. An LC50 is found when 100 percent tire crumb filtrate is prepared with 25 grams per liter of detention pond water that is collected directly after a storm. It is important to note that a natural body of water will never experience this level of tire crumb loading. It is also important to note that though the lower percentages of tire crumb filtrate did not show a significant toxic effect, the overall load is still greater than what the average detention pond will be exposed to. The area directly surrounding the filter may experience a peak in tire crumb filtrate concentration after the first storm that may expose the ecosystem to elevated concentrations. However, following the same stormwater application example using a 12 ft^3 filter with 45 percent tire crumb and a detention pond with pool permanent volume of 12 acre-ft, even if only 1 percent of the detention pond is initially exposed to the tire crumb, it will still only be exposed to 0.43 grams of tire crumb per liter of pond water.

Recommendations

Based on the results of this research with fathead minnows, the use of tire crumb in detention pond water applications is an acceptable means of recycling tires and implementing green engineering practices. Though an LC50 of 100 percent tire crumb filtrate prepared with 25 grams per liter pond water immediately after a storm was found, tire crumb is considered safe to use with detention pond water. The LC50 found is significantly higher than what can be expected in the environment and is therefore considered non-threatening to aquatic fish.

Future Research

Testing to determine chronic or long term toxicity could be more beneficial than knowing the acute toxicity. Additional research should be conducted to determine how much crumb rubber actually makes its way into local bodies of water. Lacking this knowledge, it is difficult to determine the toxic impact that may be experienced. If more is known in this regard, a more precise toxicity test may be performed compared to a worst-case scenario. However, it is highly improbable that leachate from 25 grams per liter can reach a water body and retain that concentration.

In order to get a representative impact on different species for the toxicity of tire crumb, additional testing is necessary. Generalizing toxicity based on one species may have negative implications, therefore it is suggested that research be done to determine potential toxicity to other aquatic biota, such as plants, as well as other animal species.

APPENDIX A: TOXICITY TEST DATA SHEET

Toxicant: Tire Crumb and Cypress Sawdust
Leachate: Distilled Water
Test Period:
Beginning: Date 01/30 Time 1:00
Ending: Date 02/03 Time 1:00
Test Organisms:
Species: Fathead minnows
Age: 6 days
Source: Marinco

Conc (%)	Aquarium Number	Dissolved Oxygen 0	24	48	72	96	pH 0	24	48	72	96	Number of Live Organisms 0	24	48	72	96	
Control	1A	5.6	6.1	6.3	-	-	4.7	6.0	5.3	-		10	6	0	-	-	0% Survival
	1B	6.8	5.5	6.3	6.3	-	5.6	7.0	4.7	4.9		10	7	1	0	-	
	1C	6.9	6.2	6.6	-	-	5.3	6.0	4.3	-		10	6	0	-	-	
6.25% Tire Crumb Filtrate	2A	6.9	6.9	-	-	-	5.5	0.0	-	-		10	0	0	-	-	0% Survival
	2B	6.4	6.5	-	-	-	5.2	0.0	-	-		10	0	0	-	-	
	2C	6.2	6.6	-	-	-	5.7	0.0	-	-		10	0	0	-	-	
3.125% Tire Crumb Filtrate	3A	5.6	6.7	-	-	-	5.1	0.0	-	-		10	0	0	-	-	0% Survival
	3B	6.5	6.6	-	-	-	5.5	0.0	-	-		10	0	0	-	-	
	3C	5.9	6.7	-	-	-	5.6	0.0	-	-		10	0	0	-	-	
75% Sawdust Filtrate	4A	6.8	6.3	5.9	5.6	5.3	5.6	10.0	5.7	5.7		10	10	10	10	10	97% Survival
	4B	6.4	6.6	6.1	5.9	5.9	6.0	10.0	5.7	5.4		10	10	10	10	9	
	4C	6.8	6.6	6.2	5.8	6.2	6.1	10.0	6.0	6.1		10	10	10	10	10	
50% Sawdust Filtrate	5A	6.8	6.7	6.1	5.5	6.6	6.2	10.0	6.3	6.1		10	10	10	10	10	90% Survival
	5B	6.9	6.4	6.4	6.1	6.5	6.3	10.0	6.2	6.1		10	10	10	10	9	
	5C	6.9	6.6	6.5	6.1	6.4	6.4	9.0	6.3	6.3		10	9	9	0	8	
6.25% Tire Crumb and 6.25% Sawdust	6A	6.2	6.4	-	-	-	6.0	0.0	-	-		10	0	0	-	-	0% Survival
	6B	6.4	6.7	-	-	-	6.3	0.0	-	-		10	0	0	-	-	
	6C	6.6	5.9	-	-	-	6.2	0.0	-	-		10	0	0	-	-	

Toxicant: Tire Crumb and Cypress Sawdust
Leachate: Distilled Water
Test Period:

Beginning: Date 02/12 Time 1:00
Ending: Date 02/16 Time 1:00

Test Organisms:

Species: Fathead minnows
Age: 6 days
Source: Marinco

Conc	Aquarium	Dissolved Oxygen					pH					Number of Live Organisms					
(%)	Number	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	
Control	1A	5.7	6.1	6.9	5.7	6.3	6.0	4.5	6.0	5.8	5.9	10	10	7	1	0	0% Survival
	1B	6.3	6.5	6.7	6.0	6.6	6.1	5.8	6.0	6.1	6.0	10	9	4	1	0	
	1C	6.3	6.7	6.9	5.4	-	6.1	5.7	6.2	5.8	-	10	10	5	0	-	
3.125% Tire Crumb Filtrate	2A	6.6	6.1	-	-	-	6.1	5.2	-	-	-	10	0	-	-	-	0% Survival
	2B	6.3	6.4	-	-	-	5.9	5.8	-	-	-	10	0	-	-	-	
	2C	6.7	6.4	-	-	-	6.0	5.2	-	-	-	10	0	-	-	-	
1.5% Tire Crumb Filtrate	3A	5.9	5.9	-	-	-	5.7	5.1	-	-	-	10	0	-	-	-	0% Survival
	3B	6.7	6.`	-	-	-	5.8	5.3	-	-	-	10	0	-	-	-	
	3C	6.6	6.1	-	-	-	5.8	4.5	-	-	-	10	0	-	-	-	
100% Sawdust Filtrate	4A	6.3	5.9	6.1	6.1	6.1	6.2	5.9	5.8	5.8	5.8	10	10	10	10	10	100% Survival
	4B	6.7	5.9	6.3	6.4	6.2	6.3	6.0	6.0	6.2	6.1	10	10	10	10	10	
	4C	6.6	6.4	6.5	6.2	6.3	6.5	6.2	6.3	6.1	6.2	10	10	10	10	10	
EMPTY	5A																
	5B																
	5C																
3% Tire Crumb and 3% Sawdust	6A	6.6	6.9	6.9	6.5	6.7	7.1	6.7	7.1	6.5	6.8	10	2	1	1	1	3% Survival
	6B	6.7	6.9	6.9	-	-	6.9	6.9	6.9	-	-	10	2	0	-	-	
	6C	5.9	5.9	6.6	-	-	7.0	6.7	6.7	-	-	10	3	0	-	-	

Toxicant: Tire Crumb
Leachate: Distilled Water with Buffer
Test Period:
Beginning: Date 02/26 Time 1:00
Ending: Date 03/01 Time 1:00
Test Organisms:
Species: Fathead minnows
Age: 6 days
Source: Marinco

Conc (%)	Aquarium Number	Dissolved Oxygen					pH					Number of Live Organisms					
		0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	
Control	1A	6.3	6.2	5.5	6.1	5.4	7.3	7.0	7.2	7.2	7.2	10	2	2	2	2	20% Survival
	1B	6.2	5.8	5.4	5.5	5.5	7.4	7.3	7.3	7.3	7.3	10	4	3	3	2	
	1C	6.1	5.9	5.7	5.9	5.6	7.3	7.3	7.3	7.3	7.3	10	5	3	2	2	
6.25% Tire Crumb Filtrate	2A	6.1	6.1	6.0	6.0	5.7	7.4	7.3	7.3	7.3	7.3	10	4	4	3	3	33% Survival
	2B	6.4	6.1	6.1	6.1	5.9	7.4	7.2	7.4	7.3	7.3	10	2	2	2	2	
	2C	6.6	6.1	5.7	6.0	5.8	7.3	7.3	7.0	7.2	7.2	10	9	8	7	5	
3.125% Tire Crumb Filtrate	3A	6.5	5.9	5.7	5.8	5.9	7.3	7.0	7.1	7.1	7.2	10	4	3	2	2	27% Survival
	3B	6.6	6.1	5.9	5.9	5.8	7.3	7.3	7.3	7.3	7.3	10	7	6	6	4	
	3C	6.5	5.8	5.8	6.1	5.7	7.4	7.4	7.2	7.4	7.4	10	3	3	2	2	
1.56% Tire Crumb Filtrate	4A	6.4	6.0	6.0	6.2	5.4	7.4	7.4	7.4	7.3	7.3	10	6	5	4	3	30% Survival
	4B	6.5	6.1	5.9	5.5	6.1	7.4	7.4	7.4	7.3	7.3	10	3	3	3	2	
	4C	6.4	6.1	5.8	5.7	5.9	7.4	7.3	7.4	7.3	7.3	10	5	5	5	4	
0.78% Tire Crumb Filtrate	5A	6.3	5.9	5.7	5.8	6.0	7.6	7.4	7.5	7.4	7.4	10	2	2	2	2	13% Survival
	5B	6.2	6.1	6.2	6.0	5.8	7.6	7.5	7.5	7.4	7.4	10	2	2	1	1	
	5C	6.1	6.0	6.1	5.3	5.7	7.5	7.5	7.4	7.4	7.4	10	3	2	2	1	
EMPTY	6A																
	6B																
	6C																

Toxicant: Tire Crumb and Cypress Sawdust
Leachate: Tap Water with Buffer
Test Period:
Beginning: Date 03/05 Time 1:00
Ending: Date 03/09 Time 1:00
Test Organisms:
Species: Fathead minnows
Age: 13 to 27 days
Source: Marinco

Conc (%)	Aquarium Number	Dissolved Oxygen 0	24	48	72	96	pH 0	24	48	72	96	Number of Live Organisms 0	24	48	72	96	
Control	1A	4.8	5.2	5.3	5.7	6.0	7.6	8.1	8.1	8.1	8.2	5	5	5	5	5	100% Survival
	1B	5.0	5.3	4.9	5.7	5.9	7.7	8.1	8.2	8.3	8.4	5	5	5	5	5	
	1C	5.1	5.5	5.1	5.7	5.7	7.8	8.1	8.2	8.4	8.4	5	5	5	5	5	
6.25% Tire Crumb Filtrate	2A	5.1	5.1	5.2	5.8	5.8	7.8	8.1	8.2	8.4	8.3	5	5	5	5	5	100% Survival
	2B	4.9	5.0	5.5	5.8	5.8	7.8	8.1	8.2	8.3	8.4	5	5	5	5	5	
	2C	5.0	5.2	5.7	5.8	5.7	7.8	8.1	8.2	8.4	8.5	5	5	5	5	5	
3.125% Tire Crumb Filtrate	3A	5.1	5.2	5.6	5.9	5.8	7.8	8.1	8.2	8.4	8.5	5	5	5	5	5	100% Survival
	3B	5.1	5.2	5.5	5.9	5.8	7.9	8.1	8.2	8.4	8.5	5	5	5	5	5	
	3C	5.0	5.1	5.6	5.9	5.7	7.9	8.1	8.2	8.4	8.5	5	5	5	5	5	
1.56% Tire Crumb Filtrate	4A	5.2	5.3	5.6	5.8	6.0	7.9	8.1	8.2	8.4	8.4	5	5	5	5	5	100% Survival
	4B	5.2	5.2	5.7	5.9	5.9	7.9	8.1	8.2	8.4	8.4	5	5	5	5	5	
	4C	5.1	5.3	5.7	5.9	5.8	7.9	8.1	8.3	8.4	8.5	5	5	5	5	5	
0.78% Tire Crumb Filtrate	5A	5.0	5.1	5.8	5.7	6.1	7.9	8.1	8.2	8.4	8.4	5	5	5	5	5	100% Survival
	5B	5.0	5.0	5.7	5.6	6.0	7.9	8.0	8.2	8.3	8.3	5	5	5	5	5	
	5C	5.1	5.1	4.8	5.3	5.7	7.9	7.8	8.2	8.3	8.3	5	5	5	5	5	
EMPTY	6A																
	6B																
	6C																

Toxicant: Tire Crumb
Leachate: Tap Water with Buffer
Test Period:
Beginning: Date 03/10 Time 1:00
Ending: Date 03/14 Time 1:00
Test Organisms:
Species: Fathead minnows
Age: 6 days
Source: Marinco

Conc (%)	Aquarium Number	Dissolved Oxygen					pH					Number of Live Organisms					
		0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	
Control	1A	5.8	5.3	5.5	5.1	5.0	8.0	8.3	7.9	8.4	8.4	10	10	10	10	10	97% Survival
	1B	5.6	5.3	5.6	5.2	5.1	8.0	8.3	8.1	8.5	8.5	10	10	10	10	9	
	1C	5.7	5.2	5.0	5.2	5.0	8.1	8.3	8.3	8.5	8.5	10	10	10	10	10	
12.5% Tire Crumb Filtrate	2A	5.7	5.6	5.3	4.5	5.2	8.0	8.3	8.3	8.4	8.5	10	10	10	10	10	100% Survival
	2B	5.3	5.5	5.1	4.7	5.1	8.0	8.2	8.3	8.4	8.5	10	10	10	10	10	
	2C	5.3	5.9	5.2	4.8	5.1	8.1	8.3	8.3	8.4	8.5	10	10	10	10	10	
6.25% Tire Crumb Filtrate	3A	5.8	5.7	5.4	4.5	4.7	8.1	8.3	8.3	8.4	8.5	10	10	10	10	10	100% Survival
	3B	5.6	5.5	5.5	4.5	4.6	8.1	8.3	8.4	8.5	8.5	10	10	10	10	10	
	3C	5.4	5.4	4.9	4.7	4.9	8.1	8.3	8.4	8.4	8.5	10	10	10	10	10	
3.125% Tire Crumb Filtrate	4A	5.4	5.3	5.0	4.4	5.0	8.1	8.3	8.4	8.5	8.5	10	10	10	10	9	83% Survival
	4B	5.5	5.1	5.1	4.5	5.1	8.1	8.3	8.4	8.3	8.5	10	10	10	8	8	
	4C	5.8	5.1	4.8	4.4	5.3	8.1	8.3	8.4	8.5	8.5	10	10	10	10	8	
1.56% Tire Crumb Filtrate	5A	5.7	5.2	4.9	4.5	4.9	8.1	8.4	8.4	8.5	8.5	10	10	9	9	9	97% Survival
	5B	5.6	5.3	5.3	4.5	4.8	8.1	8.3	8.3	8.4	8.5	10	10	10	10	10	
	5C	5.6	5.3	5.1	4.6	5.0	8.1	8.3	8.4	8.1	8.5	10	10	10	10	10	
EMPTY	6A																
	6B																
	6C																

Toxicant: Tire Crumb
Leachate: Tap Water with Buffer
Test Period:
Beginning: Date 03/16 Time 1:00
Ending: Date 03/20 Time 1:00
Test Organisms:
Species: Fathead minnows
Age: 6 days
Source: Marinco

Conc (%)	Aquarium Number	Dissolved Oxygen 0	24	48	72	96	pH 0	24	48	72	96	Number of Live Organisms 0	24	48	72	96	
Control	1A	4.9	4.4	4.4	4.5	4.9	7.7	8.1	8.0	8.1	8.1	10	10	9	9	9	93% Survival
	1B	4.9	4.4	4.8	4.6	4.9	8.0	8.2	8.1	8.2	8.3	10	10	10	10	9	
	1C	4.8	4.4	4.7	4.4	5.0	8.0	8.3	8.2	8.2	8.4	10	10	10	10	10	
12.5% Tire Crumb Filtrate	2A	4.9	4.5	4.8	4.2	5.1	8.0	8.2	8.2	8.2	8.4	10	10	9	9	8	83% Survival
	2B	5.0	4.6	4.9	4.3	5.2	8.1	8.4	8.3	8.3	8.3	10	10	9	9	8	
	2C	4.9	4.8	4.9	4.1	5.2	8.1	8.3	8.3	8.3	8.5	10	10	10	10	9	
18.75% Tire Crumb Filtrate	3A	4.7	4.6	4.6	4.2	5.2	7.9	8.3	8.2	8.3	8.4	10	10	9	9	9	90% Survival
	3B	4.6	4.6	4.9	4.3	5.1	8.0	8.1	8.1	8.2	8.3	10	10	10	9	9	
	3C	4.6	4.7	4.5	4.3	5.3	8.0	8.3	8.2	8.3	8.3	10	10	9	9	9	
25% Tire Crumb Filtrate	4A	4.5	4.8	4.6	4.2	5.2	8.0	8.2	8.3	8.3	8.2	10	10	8	8	8	83% Survival
	4B	4.7	4.9	4.7	4.3	5.1	8.0	8.2	8.2	8.2	8.3	10	10	8	8	8	
	4C	4.6	4.8	4.9	4.1	4.9	8.0	8.2	8.2	8.2	8.3	10	10	10	9	9	
50% Tire Crumb Filtrate	5A	4.8	4.9	4.8	4.3	4.8	8.0	8.2	8.2	8.2	8.3	10	10	10	9	9	95% Survival
	5B	4.7	4.9	4.5	4.3	5.2	8.0	8.2	8.2	8.2	8.3	10	10	10	10	10	
	5C																
EMPTY	6A																
	6B																
	6C																

Toxicant: Tire Crumb
Leachate: Tap Water
Test Period:
Beginning: Date 03/22 Time 1:00
Ending: Date 03/26 Time 1:00
Test Organisms:
Species: Fathead minnows
Age: 6 days
Source: Marinco

Conc (%)	Aquarium Number	Dissolved Oxygen					pH					Number of Live Organisms					
		0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	
Control	1A	5.2	5.9	5.7	5.3	5.3	7.9	8.1	8.5	8.3	8.0	10	4	3	3	3	23% Survival
	1B	5.3	5.2	5.1	5.2	5.2	8.3	8.4	8.5	8.5	8.2	10	1	1	1	1	
	1C	5.2	4.9	5.1	5.3	5.3	8.3	8.4	8.5	8.4	8.2	10	4	4	3	3	
12.5% Tire Crumb Filtrate	2A	5.1	5.3	5.8	5.4	5.2	8.4	8.4	8.5	8.4	8.3	10	7	5	5	5	30% Survival
	2B	5.1	5.1	5.6	5.0	5.1	8.4	8.4	8.5	8.4	8.3	10	6	2	2	2	
	2C	5.2	5.3	5.5	5.1	5.1	8.4	8.5	8.4	8.4	8.3	10	6	2	2	2	
25% Tire Crumb Filtrate	3A	5.2	5.4	5.0	5.2	5.3	8.4	8.4	8.5	8.4	8.3	10	2	1	1	1	10% Survival
	3B	5.3	5.3	5.1	5.3	5.2	8.4	8.5	8.5	8.4	8.3	10	3	0	0	0	
	3C	5.3	5.1	5.5	5.5	5.2	8.4	8.4	8.6	8.4	8.3	10	4	2	2	2	
50% Tire Crumb Filtrate	4A	5.2	4.9	5.4	5.0	5.1	8.5	8.5	8.5	8.4	8.3	10	6	6	6	6	60% Survival
	4B	5.2	5.2	5.3	5.5	5.1	8.3	8.5	8.5	8.4	8.3	10	9	8	8	7	
	4C	5.2	5.1	5.1	5.4	5.1	8.5	8.5	8.5	8.4	8.4	10	5	5	5	5	
100% Tire Crumb Filtrate	5A	5.3	5.3	5.6	4.9	5.2	8.5	8.4	8.4	8.3	8.4	10	9	9	9	9	93% Survival
	5B	5.1	5.4	5.2	5.2	5.3	8.5	8.3	8.3	8.2	8.3	10	10	10	10	10	
	5C	5.3	5.2	5.1	5.3	5.2	8.4	8.1	8.0	8.2	8.3	10	9	9	9	9	
EMPTY	6A																
	6B																
	6C																

Toxicant: Tire Crumb
Leachate: Pond Water after Large Rain
Test Period:
Beginning: Date 04/11 Time 1:00
Ending: Date 04/15 Time 1:00
Test Organisms:
Species: Fathead minnows
Age: 6 days
Source: Marinco

Conc (%)	Aquarium Number	Dissolved Oxygen					pH					Number of Live Organisms					
		0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	
Control	1A	5.1	4.8	5.1	4.4	4.3	7.8	7.8	7.3	7.6	7.9	10	10	10	10	10	100% Survival
	1B	5.1	4.9	5.2	4.3	4.3	7.9	7.9	7.8	7.9	7.9	10	10	10	10	10	
	1C	4.9	5.2	5.1	4.5	4.5	7.9	7.6	7.8	7.9	7.9	10	10	10	9	10	
12.5% Tire Crumb Filtrate	2A	4.9	5.1	4.8	4.4	4.4	7.8	7.6	7.7	7.9	7.9	10	10	10	10	10	100% Survival
	2B	4.8	5.0	4.9	4.3	4.2	7.9	7.4	7.8	7.9	7.9	10	10	10	10	10	
	2C	4.9	4.9	5.0	4.2	4.5	7.9	7.7	7.8	7.9	7.9	10	10	10	10	10	
25% Tire Crumb Filtrate	3A	5.0	5.0	5.0	4.5	4.6	7.9	7.8	7.8	7.9	7.9	10	10	10	10	10	93% Survival
	3B	5.1	4.8	4.9	4.3	4.5	7.8	7.9	7.8	7.9	7.9	10	10	10	10	9	
	3C	5.2	4.7	4.8	4.3	4.2	7.8	7.8	7.8	7.9	7.9	10	10	10	9	9	
50% Tire Crumb Filtrate	4A	5.2	4.8	4.9	4.3	4.3	7.8	7.9	7.8	7.9	7.9	10	10	10	10	8	90% Survival
	4B	4.8	5.1	4.8	4.3	4.3	7.8	8.0	7.8	7.9	7.9	10	10	10	10	10	
	4C	5.0	5.1	5.0	4.4	4.3	7.8	7.7	7.7	7.9	7.9	10	10	10	9	9	
100% Tire Crumb Filtrate	5A	4.9	5.2	5.1	4.6	4.2	7.8	7.6	7.7	7.8	7.8	10	10	9	6	3	33% Survival
	5B	4.9	5.0	5.0	4.2	4.4	7.7	7.9	7.7	7.8	7.8	10	10	10	8	3	
	5C	5.0	4.8	5.0	4.4	4.1	7.7	7.9	7.6	7.8	7.8	10	8	8	5	4	
EMPTY	6A																
	6B																
	6C																

Toxicant: Tire Crumb
Leachate: Pond Water
Test Period:
Beginning: Date 04/20 Time 1:00
Ending: Date 04/24 Time 1:00
Test Organisms:
Species: Fathead minnows
Age: 6 days
Source: Marinco

Conc (%)	Aquarium Number	Dissolved Oxygen					pH					Number of Live Organisms					
		0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	
Control	1A	4.3	4.1	4.3	4.2	4.2	7.7	7.6	7.6	7.7	7.5	10	9	9	8	8	77% Survival
	1B	4.2	4.0	4.3	4.2	4.2	7.7	7.7	7.5	7.7	7.5	10	9	9	9	8	
	1C	4.3	4.1	4.2	4.2	4.2	7.7	7.8	7.5	7.7	7.5	10	8	7	7	7	
12.5% Tire Crumb Filtrate	2A	4.3	4.1	4.2	4.2	4.2	7.7	7.8	7.6	7.7	7.5	10	8	7	7	7	80% Survival
	2B	4.3	4.2	4.1	4.1	4.2	7.7	7.8	7.6	7.7	7.5	10	10	10	9	9	
	2C	4.4	4.2	4.2	4.1	4.2	7.7	7.8	7.6	7.7	7.5	10	10	9	8	8	
25% Tire Crumb Filtrate	3A	4.4	4.0	4.2	4.3	4.2	7.7	7.8	7.6	7.7	7.5	10	8	7	7	7	67% Survival
	3B	4.0	4.2	4.2	4.3	4.2	7.7	7.8	7.6	7.7	7.5	10	9	8	7	7	
	3C	4.4	4.1	4.3	4.2	4.2	7.7	7.8	7.6	7.7	7.5	10	8	6	6	6	
50% Tire Crumb Filtrate	4A	4.3	4.1	4.3	4.2	4.2	7.7	7.8	7.7	7.8	7.5	10	8	8	8	8	73% Survival
	4B	4.2	4.1	4.3	4.2	4.2	7.7	7.8	7.6	7.7	7.5	10	9	9	9	7	
	4C	4.3	4.2	4.1	4.3	4.2	7.7	7.8	7.6	7.7	7.5	10	8	7	7	7	
100% Tire Crumb Filtrate	5A	4.3	4.2	4.2	4.3	4.2	7.7	7.8	7.6	7.7	7.5	10	9	9	7	5	57% Survival
	5B	4.3	4.1	4.2	4.3	4.2	7.7	7.8	7.6	7.7	7.5	10	8	8	8	5	
	5C	4.3	4.1	4.1	4.3	4.2	7.7	7.8	7.6	7.7	7.5	10	7	7	7	7	
EMPTY	6A																
	6B																
	6C																

APPENDIX B: SUMMARY OF EPA TOXICITY TEST GUIDELINES

Types of Tests

The test should consist of a control and a minimum of five different concentrations of what is to be tested. The results should be expressed in terms of the concentration that is lethal to fifty percent of the test organisms, which would provide a dose-response curve. If you are testing the toxicity of the receiving water, it is necessary to have a control and the undiluted receiving water, as well as a series of receiving water dilutions. The tests may be static, meaning that they test organisms are exposed to a constant test concentration, or flow through, meaning that the sample is pumped continuously to a diluter system where continuous sampling is employed. The flow through method is too costly for many operations ("Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms," 2002).

If a static non-renewal test method (the most practical and cost effective method), there are several disadvantages. For instance, depending on the type of toxicant, it may be lost through volatilization, adsorption to the tanks, or the substance could rapidly degrade. Each of these would reduce the apparent toxicity of the substance ("Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms," 2002).

Quality Assurance

According to the US EPA (2002), in order to maintain the highest level of quality of assurance when testing for toxicity, it is important that a quality assurance program be set in place. This should include the appointment of a lab quality assurance officer with the responsibility of developing and maintaining a quality assurance program. As part of this program, it is important that a written description of lab standard operating procedures be established for organism culturing, toxicity testing, instrument calibration, etc. The quality assurance program should also include all lab procedures that could affect the quality of the final toxicity data such as sample handling, condition of the specimens, condition of the equipment, instrument calibration, replication, etc.

Separate areas should be designated for test organism culturing and testing to avoid contamination of the organisms prior to testing. The test chambers shall be set up so as to maintain adequate temperature and dissolved oxygen levels. To measure the temperature of the test chambers, a thermometer must be placed directly into the test solution (United States, 2002).

The quality of water used for culturing of organisms, dilution of toxicants and in the control chamber during the experiment is extremely important. The water should consistently come from the same source.

The health of the test organisms can be assessed by the survival, growth, and reproduction of the organisms. The nutritional quality of the food is important to culture and test healthy fish and invertebrates. For the results of a test to be acceptable, control survival must greater or equal to 90 percent.

According to the EPA (2002), it is the laboratory's responsibility to demonstrate an ability to obtain both consistent and precise results with reference toxicants before attempting to perform a toxicity test on an unknown toxicant for permit compliance purposes. A minimum of five tests should be performed with a reference toxicant using different batches of test organisms.

Facility and Equipment

According to the US EPA (2002), it is very important to make sure that the culture and toxicity testing areas are kept separate. This is to ensure that fumes from the toxicants do not reach the fish being cultured. It is also important to keep the stored samples away from both the culturing tanks and the testing tanks to ensure that neither is contaminated.

In order to control the amount of light that the specimens are exposed to, it is recommended that an automatic timer be used. Air used for aeration of the tanks must not contain oil or fumes. To keep the stress level of the specimens as low as possible, they should be protected from external disturbances such as pedestrian traffic.

The preferred materials are tempered glass and TEFLON to avoid possible sorption and leaching of toxic substances. These materials may be reused after cleaning, while others may not. If a new plastic product is to be used, it should be first tested for toxicity by exposing test specimens to them under ordinary test conditions. To maintain the highest level of quality, it is important that copper, galvanized material, rubber, brass, and lead never come in contact with holding water, dilution water, or test solutions. If a silicone adhesive was used to construct the glass test chambers, any extra adhesive inside the chamber should be removed to keep the adhesive from contacting the water in the event organochlorine or organophosphorus is in the water.

If using new plasticware for dilution water collection or organism test chambers, it is only necessary to rinse the new sample containers once with sample dilution water. New glassware, on the other hand, must be soaked overnight in 10 percent acid and rinsed well with both DI and sample dilution water. However, if you are reusing a nondisposable sample container, EPA (2002) has specified the following method to clean the container:

1. Soak 15 minutes in tap water and scrub with detergent.
2. Rinse twice with tap water.
3. Rinse once with fresh, 10 percent dilute hydrochloric or nitric acid (10 mL concentrated acid with 90 mL deionized water) to remove scale, metals, and bases.
4. Rinse twice with deionized water.
5. Rinse once with full-strength, pesticide-grade acetone to remove organic compounds (use a fume hood or canopy).
6. Rinse three times with deionized water.

Even after the chambers have been cleaned thoroughly, it is still necessary to rinse each container with dilution water immediately prior to use.

It is preferable to test organisms that have been cultured in-house. However, if it is not practical to culture the organisms in house, EPA has set aside very specific shipping and handling instructions if the organisms must be acquired from a commercial source.

For feeding, the readily available TETRAMIN and BIORIL flake foods are acceptable by the US EPA (2002). However, they recommend that it only be used as a short-term substitute for feeding fathead minnows. For a long term feeding plan, No.1 pellets can be prepared according to current U.S. Fish and Wildlife Service specifications.

Test Organisms

If there is any question as to the species of the test organism, the questionable specimen should be sent to a taxonomic expert to confirm the identification. *Pimephales promelas* (fathead minnow) is one of the EPA (2002) recommended test organisms because they are easily cultured in the laboratory and are sensitive to a variety of pollutants. Whether the organisms are cultured in house or commercial purchased, they should be observed to ensure that they show no signs of stress or disease.

Because young organisms are more sensitive to toxicants, it is to test specimens while they are in the early stages of life. All organisms should be approximately the same age and acquired from the same source. In addition, to keep the test data consistent, tests should be on fish that are of approximately the same age.

To maintain the health of the fish, water should flow through an activated carbon or underground filter to remove waste. The US EPA offers other methods to clean the holding tank water, such as piping water through high intensity ultraviolet light for disinfection. The dissolved oxygen levels should be maintained at a minimum of 4.0 mg/L for warm water species and 6.0 mg/L for cold water species. Uneaten food and fecal matter should be siphoned from the bottom of the tank at least twice a week.

Fish should be observed each day and removed upon signs of disease, stress, physical damage, or mortality. A daily record should be kept documenting feeding, behavior, mortality, and other observations. In addition, when handling the specimens, they should be handled as gently and quickly as possible to minimize stress. This is easiest accomplished using dipnets. If specimens are transferred to a testing tank and appear to be unhealthy, discolored, stressed or if mortality exceeds 10 percent prior to the test, the group must not be used for testing.

Once the test is complete, the EPA (2002) suggests that all test organisms used in a toxicity test be humanely euthanized.

Dilution Water

The US EPA (2002) suggests the following method of making standard, synthetic freshwater.

1. Place 19 L of dionized water in a plastic carboy.
2. Add 1.20 g $MgSO_4$, 1.92 g $NaHCO_3$, and 0.080 g KCl to the carboy.
3. Aerate overnight.
4. Add 1.20 g $CaSO_4 \bullet 2\ H_2O$ to 1 L dionized water in a separate flask.
5. Aerate the combined solution vigorously for 24 hours to dissolve the added chemicals and stabilize the medium

The following table, adapted from US EPA (2002) can be used to prepare water based on the needs of the final water in terms of pH, hardness, and alkalinity.

Table 4: Preparation of Synthetic Freshwater Using Reagent Grade Chemicals[2]

	Reagent Added (mg/L)[2]				Approximate Final Water Quality		
	$NaHCO_3$	$CaSO_4 \bullet 2H_2O$	$MgSO_4$	KCl	pH[3]	Hardness[4]	Alkalinity[4]
Very soft	12.0	7.5	7.5	0.5	6.4-6.8	10-13	10-13
Soft	48.0	30.0	30.0	2.0	7.2-7.6	40-48	30-35
Moderately Hard	96.0	60.0	60.0	4.0	7.4-7.8	80-100	57-64
Hard	192.0	120.0	120.0	8.0	7.6-8.0	160-180	110-120
Very hard	384.0	240.0	240.0	16.0	8.0-8.4	280-320	225-245

Using tap water as a dilution is frowned upon, however if necessary, the EPA offers a method to dechlorinate and fully treat the water.

Acute Toxicity Test Procedures

The dissolved oxygen concentration in the samples should be near saturation prior to use. If necessary, aeration can be used. However, if testing the toxicity of volatile chemicals, aeration should be minimized. The pH should be kept within 6.0 and 9.0 to prevent mortality due to high pH. Freshwater samples are generally adjusted to a pH of 7.0 prior to testing. Two parallel tests could be run, one without an adjusted pH and one with an adjusted pH.

[2] Acquired from US EPA (2002), Table 7.

Effluent concentrations of 6.25%, 12.5%, 25%, 50%, and 100% are commonly used in toxicity testing, which should be kept in mind when determining what concentration of the chemicals in mind should be used. According to the EPA (2002), each concentration must be tested on a minimum of 20 organisms. It is also specified that each concentration and control should have a minimum of two test chambers to allow for easier viewing and counting as well as ensure that each chamber is not overloaded. In terms of live fish weight, each test chamber should have no more than 1.1 g/L at 15°C, 0/65 g/L at 20°C, or 0.40 g/L at 25°C. In terms of standard aquaria practice, this roughly equates to 1 inch of fish per gallon of water.

For tests to be acceptable, approximately 90% of the organisms in the control test chambers must survive.

Table 5: Summary of Test Conditions[3]

Summary of Test Conditions and Test Acceptability Criteria for Fathead Minnow, *Pimephales Promelas,* Acute Toxicity Tests with Effluents and Receiving Waters	
Test type	Static non-renewal, static renewal, or flow-through
Test duration	24, 48, or 96 h
Temperature	20°C ± 1°C; or 25°C ± 1°C (recommended). Test temperatures must not deviate by more than 3°C during the test (required)
Light quality	Ambient laboratory illumination (recommended)
Light intensity	10-20 µE/m2/s (50-100 ft-c) (ambient laboratory levels) (recommended)
Photoperiod	16 h light, 8 h darkness (recommended)
Test chamber size	250 mL (recommended minimum)
Test solution volume	200 mL (recommended minimum)
Renewal of test solutions	After 48 hours (required minimum)
Age of test organisms	1-14 days; less than or equal to 24-h range in age (required)
No. organisms per test chamber	10 for effluent and receiving water tests (required minimum)
No. replicate chambers per concentrations	2 for effluent tests (required minimum) 4 for receiving water tests (required minimum)
Feeding regime	*Artemia* nauplii are made available while holding prior to the test; add 0.2 mL *Artemia* nauplii concentrate 2 h prior to test solution renewal at 48 h (recommended)
Test chamber cleaning	Cleaning not required
Test solution aeration	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min (recommended)
Dilution water	Moderately hard synthetic water, receiving water, ground water, or synthetic water, modified to reflect receiving water hardness (available options)
Test concentrations	Effluents: 5 and a control (required minimum)
Dilution series	Effluents: ≥0.5 dilution series (recommended)
Endpoint	Mortality (required)
Sampling and sampling holding requirements	Receiving Waters: Grab or composite sample first used within 36 h of completion of the sampling period (recommended)
Sample volume required	2 L for effluents and receiving waters (recommended)
Test acceptability criterion	90% or greater survival in controls (required)

[3] Adapted from US EPA (2002)

REFERENCES

American Public Health Association. (2005). *Standard methods for the examination of water and wastewater* (11th ed.). New York: American Public Health Association, American Water Works Association, Water Pollution Control Federation.

Day, K.E., Holtze, K.E., Metcalfe-Smith, J.L., Bishop, C.T., & Dutka., B.J. (1993). Toxicity of leachate from automobile tires to aquatic biota. *Chemosphere*, *27*, 665-675.

Global Tire Recycling (1998). Tire Crumb Manufacture Specifications. Wildwood, FL.

Groenevelt, P.H., & Grunthal., P.E. (1998). Utilization of crumb rubber as a soil amendment for sports turf. *Soil and Tillage Research, 47*, 169-172.

Gualtieri, M., Andrioletti, M., Vismara, C., Milani, M., & Camatini, M. (2005). Toxicity of tire debris leachates. *Environmental International. 31,* 723-730.

Gunasekara, A.S., Donovan, J., & Xing, B. (2000). Ground discarded tires remove naphthalene, toluene, and mercury from water. *Chemosphere. 41*, 1155-1160.

Hansson, S.O., & Ruden, C. (2006). Evaluating the risk decision process. *Toxicology*, *218*, 100-111.

Jan, J., Yoo, T., Oh, J., & Iwasaki., I. (1998). Discarded tire recycling practices in the United States, Japan and Korea. *Resources, Conservation and Recycling. 22*, 1-14.

Khan, N., Mostafa, A., Luk, W., & Luk, G. (1995). A Comparison of Acute Toxicity of Biodiesel, Biodiesel Blends, and Diesel on Aquatic Organisms. *Journal of the Air & Waste Management Association*, 286-96.

Lisi, R.D., Park, J.K., & Stier, J.C. (2004). Mitigating nutrient leaching with a sub-surface drainage layer of granulated tires. *Waste Management. 24*, 831-839.

Martins, J., Teles, L.O., & Vasconcelos, V. (2004). The effects of environmental pollutants on complex fish behavior: integrating behavioural and physiological indicators of toxicity. *Aquatic Toxicology, 68*, 369-392.

McCarty, L.S. (2002). Issues at the interface between ecology and toxicology. *Toxicology, 181-182*, 497-503.

Ryan, P. (2008). Reducing effluent phosphorus and nitrogen concentrations in a wet detention pond using chamber upflow filter and skimmer.Title (Masters thesis, University of Central, 2008).

Scott, G.R. & Sloman, K.A. (2004). The effects of environmental pollutants on complex fish behavior: integrating behavioural and physiological indicators of toxicity. *Aquatic Toxicology, 68*, 369-392.

Tang, Z., Butkus, M.A. and Xie, Y.F. (2006). Crumb rubber filtration: A potential technology for ballast water treatment. *Marine Environmental Research, 61*, 410-423.

Teather, K., & Parrott, J. (2006). Assessing the Chemical Sensitivity of Freshwater Fish Commonly Used in Toxicological Studies. *Water Quality Research Journal of Canada, 41*, 100-5.

Toxicity Testing. (2006). Marinco Bioassay Laboratory. Retrieved April 13, 2007. <http://www.biologylab.com/ index.php>.

U.S. Environmental Protection Agency. (2002) *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms.* Washington: Office of Water. <http://www.epa.gov/WET/disk2/>

U.S. Environmental Protection Agency. (2006). *LC50*. <http:// www.epa.gov/ceampubl/fchain/lc50/>.

U.S. Environmental Protection Agency. (2007). *Ground Rubber Applications*. < http://www.epa.gov/garbage/tires/ground.htm>.

Wik, A., & Dave, G. (2006). Acute toxicity of leachates of tire wear material to *Daphnia magna*-Variability and toxic components. *Chemosphere, 64*, 1777-1784.

Wik, A., & Dave, G. (2005). Environmental labeling of car tires-toxicity to *Daphnia magna* can be used as a screening method. *Chemosphere, 58*, 645-651.

Xie, Y. (2007). Filter media: Crumb rubber for wastewater filtration. *Filtration and Separation, 44,* 30-32.