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Impact of Microsparticle Concentration Levels upon Toxicity of Phenol to Artemia

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IMPACT OF MICROPARTICLE CONCENTRATION LEVELS UPON TOXICITY OF PHENOL TO *ARTEMIA*

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ABSTRACT

Plastic pollution constitutes a threat to marine wildlife because of the deleterious impacts ranging from entanglement to ingestion of plastic debris. However, knowledge regarding the impacts of fragmented plastics into micron sizes and their interaction with other toxicants in the marine environment is still limited. In the present study the impact of polystyrene microspheres, 3 µm in diameter, upon toxicity of phenol to the brine shrimp *Artemia* was investigated in acute toxicity tests. The brine shrimp are employed as a model organism in marine toxicity tests. Phenol is a hydrophobic compound used as an intermediate resin discharged to the environment. Adult brine shrimps reared in the laboratory were exposed to phenol at nominal concentrations ranging from 40 to 200 mgL^{-1} to quantify the toxicity of phenol. Polystyrene microspheres at nominal concentrations of 100, 200 and 300 mgL^{-1} were then loaded to the phenol solutions to examine their impact upon toxicity of phenol to the brine shrimp. Results suggested that toxicity of phenol, as expressed by LC50 values, was lowered by the addition of higher concentrations of microspheres to test solutions for 48-h and 72-h exposure times. The data suggest that sorption of phenol to polystyrene beads is supported by other plastic congener profiles, protecting the brine shrimp against toxic levels of phenol. Moreover, the body burden of beads was increased with the increase of bead concentration, reveling that more beads in the organism can interact with the chemical.

DEDICATION

The present thesis is dedicated to my parents, Carlos and Lucia; my brother, Carlos Vicente, from Guayaquil-Ecuador and to all the people who have been involved directly and indirectly in achieving my goal to pursue graduate studies overseas.

I also would like to dedicate this work to the Hispanic graduate students, in general, and to the Ecuadorian graduate students, in particular, in the U.S. whose hard work, dedication, talent and determination acknowledged by the Scientific Community keep inspiring the new generations to come.

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CHAPTER ONE

BACKGROUND

Plastics

In the last century petroleum-based synthetic polymers have become indispensable materials of consumer products. Synthetic polyesters such a poly (ethylene terephthalate) or PET is a developed material, now used as a major material for manufacturing plastic-filled composites. Plastics are synthetic polymers that can be classified in many ways, but most commonly by their polymer backbone: polyvinyl chloride, polymethyl methacrylate, and other acrylics, silicones, polyurethanes, etc. Synthetic plastics have been extensively used in thousands of engineering applications. However, approximately 30% of the plastics are used today for packing applications. Sabir (2004) reported that plastic utilization is expanding at a rate of 12% per year. The combination of physical and chemical properties of plastics such as tensile strength, light weight, high thermal stability, durability, and microbial and environmental degradation over naturally occurring materials such as wood or aluminum have contributed to the widespread market of plastics. Global plastics production and consumption have been increased over the last 60 years from 1.5 million tons in 1950 to total global production of 245 million tons in 2008 (Plastics Europe, 2008).

Environmental challenges of plastic pollution

Plastic pollution has been documented on quantities and visibility in litter in natural terrestrial, freshwater and marine habitats. For example, surveys on beaches are used to monitor the accumulation of stranded plastics and other type of litter in a specific area at a certain time (Frost and Cullen, 1997; Kursui and Noda, 2003; Ribic, 1998; Walker et al., 1997). Although beach surveys provide estimates of abundance of visible plastics, there is an exclusion of the small fragments of plastics (Moore et al., 2001) and of items being transported into streams and rivers by storm-water runoff and flood events (Thompson et al., 2005). The literature on plastic pollution largely focuses on marine systems because many of these plastic wastes from different sources enter the sea as ultimate fate. The United Nations Environment Program (UNEP) estimates 6.4 million tons of litter normally ends up in the sea annually.

In the same way, pollution by plastic debris in marine environment represents deleterious environmental effects over the biological diversity (Moore et al., 2001). In marine environment any manufactured solid waste that enters the sea has been defined as marine debris (Coe and Roger, 1997). Plastic materials and products such as discarded fishing nets, ship-based release and offshore installations that end up in the marine environment are linked to potential sources of marine debris (Williams et al., 2005; Redford et al., 1997; Shiber, 1979; Gregory, 1978). The principal component of floating marine debris is plastics (Coe and Rogers, 1997; Derraik, 2002). In the Mediterranean Sea, plastics compromise about 80% of floating debris, and in the SE Pacific off the Chilean coast (Thiel et al., 2003; Morris, 1980). A survey study in the Kuroshiro Current

in NW Pacific Ocean by Yamashita and Tanimura in 2007 reported that >55% of the monitored stations contained fragments of plastic products and plastics sheets.

Environmental challenges of microplastic particles

The presence of marine plastic debris in the sea presents deleterious impacts on marine biota (Goldberg, 1995). The two main threats to marine life are entanglement and ingestion of plastic debris linked to packing bands, synthetic ropes and lines, or fishing nets (Lasit, 1997; Quayle, 1992). Species such as turtles, fish, seabirds and mammals have been reported to ingest or become entangled in plastic debris, resulting in impaired movement and feeding, reduced reproductive output, lacerations, ulcers and death (Gregory, 2009; Derraik, 2002; Laist, 1997). However, the degradation of large plastic articles through a combination of photodegradation, oxidation and mechanical abrasion pose another threat to marine environments (Andrady, 2003).

Degradation processes fragment plastics into a wide array of particles size ranging from large, >5 mm, to microscopic, <1 μm (Cheshire at al., 2009; Arthur at al., 2009). Moreover, alternative routes for microplastics to enter the sea include the direct release of small components of plastics used as abrasives in industrial and domestic cleaning applications, and spillage of plastic pellets and powders employed for the manufacturing of most plastic products (Barnes at el., 2009). Archived plankton samples from waters of the northeast Atlantic have demonstrated that abundances of microscopic plastics in the water column from this area has increased considerably over the last 40 years, in relation with the increased rise in global plastic production (Thompson et al., 2005).

Besides laboratory experiments have shown the ingestion of 10 µm polystyrene microspheres for a wide class of marine organisms that includes polychaetes, echinoderms, bryozoans and bivalves (Ward and Shumway, 2004). Similarly, Browne et al. (2008) have also demonstrated that mussels retain microplastics for over 48 days. However, the extent and consequences of ingestion of microplastics by natural population is still limited.

The ability of microplastic particles to concentrate low-level hydrophobic contaminates from seawater and to transfer them into the marine food chain poses the threat of bioconcentration of these compounds. Particularly, the translocation toxic substances to higher level organisms through seafood consumption, ultimately, human populations. The potential for plastics to transport hydrophobic contaminants was examined in a sediment-dwelling organism, *Arenicla marina*, using three microplastics and phenanthrene as toxic compound. Results showed that chemical sorption to plastics greatly exceeded sorption to natural sediments, suggesting that plastics may be important agents in the transport of toxic substances found in the environment (Teuten et al., 2007). However, the effects of microplastics on the different levels of food chain are still poorly understood.

Marine Organism Model

Crustaceans of the genus *Artemia* are in the phylum Arthropoda of the class Crustacea. Species of the genus *Artemia* (brine shrimps) are characterized by its adaptability to hypersaline environments. The genus is composed of parthenogenetic and

bisexual species. *Artemia franciscana franciscana* is an example of a bisexual species. For taxonomic purposes, criteria such as morphology of adults, specific numbers of chromosomes, genetic distance and crossbreeding experiments have been used extensively (Abreu-Grobois, 1987).

The brine shrimp Artemia: general features

The *Artemia* development has been well documented in the literature. Its life cycle begins as cysts, then emerged embryos, nauplii, finally larvae and adult (Anderson, 1967; Sato, 1967). Cysts are encased in hard, protective shell. Here, the embryo is metabolically inactive (Sorgeloos, 1980). Cysts are spherical, averaged 250-350 µm in diameter. In marine aquaculture hatcheries the cyst shell or chorion is treated chemically (known as decapsulation) before exposing cysts to hatching conditions. The reasons to decapsulate cysts include speeding up the hatching process, improving hatchability, makes it easier for the nauplii to emerge and disinfecting cysts when their origin is unknown (Léger et al., 1987; Sorgeloos et al., 1986). Upon breaking of the cyst the free-swimming larva emerges. Naupliar stages include 15 molts before reaching the adult stage. The first is Instar I (400-500 µm). The hatched nauplii subsist on yolk and stored reserves. The following Instars (2-15) or Metanauplii development, nauplii begin to their exogenous feeding. Although the duration varies depending on the *Artemia* strain Metanauplii development lasts between 24 and 36 hours and nauplii average 500 µm in length (Sorgeloos, 1980; 1976; Nimura, 1967).

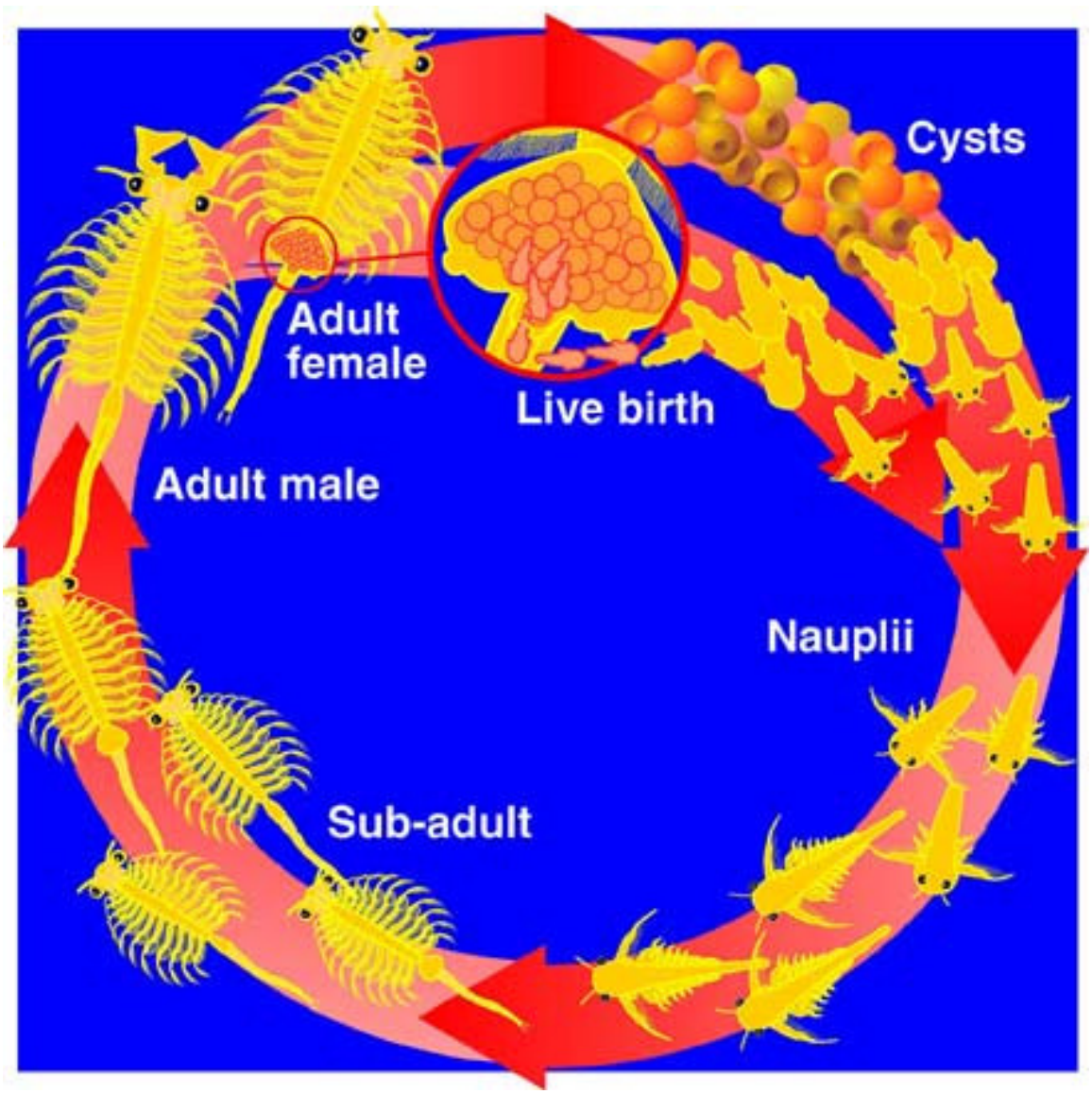


Figure 1.1: Schematic of *Artemia* life cycle
Source: U.S. Geological Survey

Literature reports *Artemia* as euryhaline and eurythermal crustacean with a short generation time (average 3 weeks), a high fecundity rate (about 100 offsprings every 4 days) and an estimated long lifespan up to more than 3 months (Ivleva, 1969; Nimura, 1967). *Artemia* populations present two modes of reproduction, depending on

environmental conditions, when these conditions are favorable they are ovoviviparous but when conditions of the water are such high salinity or low oxygen levels, oviparity is developed and embryos are released as eggs by females (Sorgeloos, 1980; Nimura, 1967). On the ovoviviparous reproduction, nauplii hatch in the ovisac of the mother and they are born alive. In contrast, the oviparous reproduction yields embryos physiologically developed up to the gastrula stage covered by a thick shell (Nimura, 1967).

Ecology and natural distribution

The habitats of the genus *Artemia* have been mainly identified in brine localities where anionic composition is concentrated by climatic conditions and altitude (Whitaker, 1940), however *Artemia* individuals have a wide geographical distribution (Browne, et al., 1984; Sorgeloos, 1980). These environments included places of marine origin (thalassohaline). For example, natural *Artemia* populations are abundant in the mono lakes (e.g, Great Salt Lake, Utah, U.S.; Mono Lake, California, U.S.), chloride lakes and sulfate lakes where the relative composition of anions, carbonate, sulfate and chloride can produce complex anionic waters, and even more saline waters than the sea and where other life forms are barely developed (Triantaphyllidis et al., 1998; Rawson and Moore, 1944). *Artemia* can be found in nature at salinity levels ranged from 60 to 220 g L^{-1} (Vanhaecke and Sorgeloos, 1980). The wide distribution of *Artemia* is also achieved due to tolerance to other environmental factors such as efficient osmoregulatory system, low

oxygen levels and production of dormant embryos (cysts) that are common to different biotopes of *Artemia* (Amat, 1985; Whitaker, 1940).

Members of the genus *Artemia* are non-selective filter feeders. They filter feeding on microalgae, bacteria and detritus. In nature *Artemia* populations constitute part of simple trophical structures and low species diversity. Moreover, the fluctuating conditions with regard to temperature, salinity and ionic composition limit the development of predators or food competitors (Sorgeloos, 1980; Sorgeloos et al., 1977). Although the absence of ecological predators allow *Artemia* biotopes to develop in great numbers in monocultures by using bacteria, protozoa and algae as diet resources (Amat, 1985), natural *Artemia* still represent a source of energy for some migrating shorebirds such as flamingos and gulls in some places. These migrating birds represent natural vectors for dispersion of *Artemia* cysts to other worldwide locations since cysts can adhere to feet and feathers of birds, and through undigested feces (Sorgeloos, 1980). Finally, there are few areas (e.g., the U.S.) where the natural populations have been exploited, but the commercial *Artemia* cysts are still the most important source of brine shrimps (Sorgeloos et al., 1977).

The use of Artemia as test organism

The genus *Artemia* is one of the animal models used for toxicity tests in laboratory conditions under marine and estuarine water environments. Aspects of *Artemia* such as biology, life cycle, high reproductive capacity, continuous availability of eggs, flexibility for nutrient sources, temperature and salinity tolerance, adaptability to

laboratory conditions and low cost of maintenance/test conditions contribute to its use as test organism in bioassays (Nunes, et al., 2006). The use of *Artemia* as species for research purposes in toxicology has been reported for a wide variety of contaminants, such as insecticides and s-triazine herbicides (Varó et al., 2002a,b; Song and Brown, 1998), mycotoxins (Barahona-Gomariz et al., 1994; Hlywka et al., 1997), toxic cyanobacteria (Beattie et al., 2003), metals (Brix et al., 2003; Hadjispyrou et al., 2001), pharmaceuticals (Nunes et al., 2005; Parra et al., 2001; Touraki et al., 1999) and organic solvents (Barahona-Gomariz et al., 1994). *Artemia* as sensitive species has been also used in ecotoxicity tests to assess the toxicity of phenolic compounds and industrial effluents. Guerra (2001) and Walker (1988) conducted toxicity studies to estimate the relative sensitivities of different aquatic invertebrates to phenol. The 24h LC50 values reported for *Artemia* were 17.35 mg L^{-1} and 56 mg L^{-1}, respectively for each study. Similarly, Price et al. (1974) conducting studies on fresh and seawater systems to evaluate biodegradability and toxicity of over 50 synthetic organic compounds of major commercial importance used *Artemia* in the toxicity bioassays and reported its sensitivity to phenol as 154 mg L^{-1} for the 24h LC50 and 57 mg L^{-1} for the 48h LC50.

As for aquatic Ecotoxicology, Hadjispyrou et al. (2001) evaluated the possibility of heavy metal bioaccumulation in marine invertebrates, including the *Artemia* genus. Their results showed that *Artemia franciscana* can be used in bioaccumulation studies. Likewise, Varó et al. (2000) performed studies on *Artemia* as potential bioaccumulation vector of chlorpyrifos due to its position in the food chain as primary consumer. Moreover based on studies conducted by Petrucci et al. (1995), *Artemia* as the main

component of zooplankton in natural salt environments in Sardinia (Italy) was a suitable bioindicator for trace elements and could reflect bioaccumulation through the food chain.

Adult *Artemia* individuals were selected as animal models to conduct acute toxicity tests (24 h, 48 h and 72 h) in the present study given its standardized test features such as continuously available, adaptability to laboratory testing conditions, tolerance environmental factors and suitable marine organism. Moreover, members of genus *Artemia* are non-selective filter-feeders that feed on a variety of microalgae, filtering particles smaller than 25 µm in size, irrespective of the particle nature (Sorgeloos and Persone, 1975). The organism filter-feeding capacity was used as an important feature for the evaluation of impact of plastic microparticle uptake during the trials proposed in the study.

Synthetic organic compounds: phenolics

Phenolics are both naturally produced by aquatic and terrestrial vegetation and by manufacturing processes such as coking of coal, chemical plants (pesticides), wood preserving and oil refineries. These chemicals are organic compounds consisting of a hydroxyl group (-OH) bonded to an aromatic hydrocarbon group. Functional groups categorized are halo-, chloro-, nitro-, amino-, alkyl- and polyhydric phenols. Degradation products from pesticides such as TFM (3-trifluoromethyl-4-nitrophenol) and Carbaryl® (1-naphthyl-methylcarbamate) are also included as phenolics (Buikema, 1979). Their volatility generally decreases with increasing molecular weight. The boiling point and

melting point increase with increasing substitution for chloro- and nitrophenols (EPA, 1977a).

Major chemical properties of phenolics are based on their aromatic component (benzene ring, C_6H_6). Hydrogens from the benzene ring can be replaced by other functional groups such as a methyl group (CH_3) to form methylbenzene ($C_6H_5CH_3$), or a hydroxyl group (-OH) attached to the benzene ring to from phenol (C_6H_5OH). The number of functional groups and positions attached to the aromatic component vary, placing the main group at the top of the drawing structure. Moreover the presence of the hydroxyl group gives acidity characteristics to phenolics. The acidity constant (pKa) for phenolics is reported between 10 and 12. However, pKa values tend to decease with an increase in the number of chloro or nitro groups.

The simplest member of the phenolic class is phenol. The structure of phenol is a monohydroxy derivative of benzene. Phenol molecular formula is C_6H_5OH and has a molecular weight of 94.11 (Lide, 1993). The molecular structure of phenol is shown in Figure 2. The major uses of phenol include as an intermediate in the production of phenolics resins and in the production of bisphenol A. Phenol is also used as a disinfectant and in numerous medical preparations (e.g. antiseptic, anesthetic). Its wide-range of uses makes phenol rank in the top in chemical production in the U.S. In 2004, the U.S. total annual capacity of phenol production approached 6.6 billion pounds (CMR, 2005).

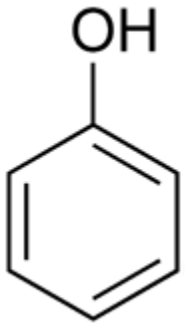


Figure 2.1: Structure of phenol

The physical properties describe pure phenol as crystalline solid with a distinct odor that is sickeningly and acrid (HSDB, 2006). Melting and boiling points for phenol are 43°C and 181.1°C (Lide, 1993), respectively. Phenol has a low vapor pressure of 0.35 Torr at 25°C (HSDB, 2006).

The chemical properties report phenol as relatively soluble in water with a solubility of 87 g L^{-1} at 20°C, but phenol is very soluble in organic solvents such as alcohol, chloroform, ether, benzene and acetone (Lide, 1993). The Henry's law constant for phenol is $4.0x10^{-7}$ m^3/mol at 25°C (Lide, 1993). The tendency for lipids (biota) given by the octanol-water partition coefficient, Log Kow, for phenol is 1.46 and its carbon-water partition coefficient, Log Koc, is between 1.21-1.96 (HSDB, 2006). Phenol seems to biodegradate rapidly in water (half life of about 15 hours) and soil (half-life of 15 days), however due to its pKa of 10 phenol will primarily exist as the protonated acid at environmental pH values. Moreover, in acidic environments (e.g., acidic soils) the half-lives of phenol may be longer (ATSDR, 2006).

Phenol in aquatic systems

Total phenols and individual phenolic constituents enter solids and water through industrial and domestic waste waters and natural waters. The terrestrial inputs from industrial effluents constitute an important source of discharges of phenolics to aquatic systems. The wide variety use of phenols in manufacturing processes and the growing production contribute to the presence of phenols in the environment (CMR, 2005). As a result, phenols have been found in at least 595 of the 1,678 of the National Priority List sites identified by the Environmental Protection Agency (EPA). The TRI reported an estimated release of 85,500 pounds of phenol to surface water from 689 domestic manufacturing and processing facilities in 2004. Moreover, many industries wastes contain phenolic materials that are difficult to remove by conventional treatment methods (Rengaraj et al., 2002).

Concentrations of phenol in industrial rivers in the U.S. have been reported between 0 and 5 ppb (Sheldon and Hites, 1978; 1979). However, concentration of phenol ranged from 10 to 100 ppb has been also reported for rivers in the U.S. (Jungclaus et al., 1978). Levels of phenol become higher in sites near to industrial centers and to population centers where the use of phenol-related products is widespread. Phenol was detected at concentrations of 33.5 ppm (Pfeffer, 1979) and 100 ppb (Paterson et al., 1996) in petroleum refinery waters.

Photochemical degradation and microbial action represent two of the mechanisms in which phenol is degraded in the environment. However, exact conditions under which phenol is degraded are still in review. In lakes phenol is readily biodegradable. A studied

in river water showed complete removal of phenol after 2 days at 20°C and 4 days at 4°C (Ludzack and Ettinger, 1960). However, the degradation of phenol has been reported slower in salt water with a half-life of 9 days reported in an estuarine river (EPA, 1979b). Experience from the present study indicated that the phenol dissolved in synthetic salt water in concentrations ranging from 40 to 200 mg L^{-1} and stored in control conditions (±10 °C) can extend its half life for than a month.

The environmental fate and movement of phenols in aquatic ecosystems have been studied in pesticides that develop phenolic constituents as intermediate degradation products (e.g. Dioxin). Other works include the effects of pulp paper mill effluents on aquatic species such rainbow trout. Lyman et al. (1982) reported a volatilization half-life of 88 days for phenol evaporation in from a model river. EPA (1988) database reported an average concentration of 10 ppb (dry weight) of ambient sediment phenol in the Pacific Ocean near Los Angeles. Bioconcentration of phenol has been reported in aquatic organisms, particularly fish. Log bioconcentration factors (BCF) of 0.28 for goldfish and 1.3 for golden orfe were reported by Kobayashi et al. (1979) and Freitag et al. (1985), respectively. Nicola et al. (1987) reported the highest mean level of phenol at 0.14 ppm detected in bottom fish (Tacoma Bay, Washington). Likewise, other works have reported that phenol and phenolic compounds accumulated in lower organisms such as algae, snails, mosquito larvae and cladocerans (Buikema, et al., 1979).

Aquatic organisms affected by toxicity of phenol

The literature of phenol toxicity from microbial populations, algae, invertebrates and vertebrates (Buikema et al., 1979) report acute toxicity values ranging from 6.5 to 1840 mg L^{-1} among test organisms. The symptoms of phenol poising observed in freshwater invertebrates (e.g., *Asellus Aquaticus*), oligochaetes, insects and fish suggest a similar mode of action among species that include a phase of immobilization prior to death (Green et al., 1988; Buikema et al., 1979). Although an exact mechanism of phenol toxicity is not clearly known, it has been found that phenol is a non-specific metabolic inhibitor, interfering with enzyme function, oxygen consumption and disrupting ATP reduction by uncoupling of the oxidative phosphorylation (Buikema et al., 1979; EIFAC, 1972). Phenol toxicity is also affected by environmental factors such as temperature and salinity, pH, water hardness, and interaction with other pollutants. Key and Scott (1986) reported that exposure of the mud crab to a chlorine-phenol mixture may inhibit the uptake of phenol, possibly because of interference with the permeability of the gill. Testing toxicity of phenol to black tilapia, Saha et al. (1999) observed on fish exposed to the chemical excess mucous secretion from skin and gill, and acute respiratory distress after a 96h exposure. In general, fish are the most sensitive species and the sensitivities of marine and freshwater organisms are similar. In addition, toxicity may be less in continuous flow tests than in static toxicity tests.

Statistical analysis: methods for estimating LC50 values

LC50 is a term that stands for the median Lethal Concentration of a toxicant that kills 50% individuals of a particular group of organisms tested in controlled laboratory conditions, and it has been used extensively in toxicology studies to estimate biological responses quantitatively. The least variation in a curve depicting responses from experimental units treated is found at the 50% level of response.

The most common toxicity effect in aquatic organisms is mortality in acute toxicity tests. Acute effects are those occurring immediately as a result of a short period of exposure to a chemical. The length of exposure is usually 24h, 48h, and 72h to days. LC50 values estimate mortality at 50% as an endpoint of toxicity in short-period tests. For all the practical purposes, the application of LC50 as a method to estimate the quantitative response of adverse effects in groups of test organisms considers measured concentrations of the chemical in the exposure medium and not the nominal concentrations to yield the percentage of organisms exhibiting lethality as the defined response. Since the test organisms are usually exposed to various concentrations of the chemical for a specific period of time, a concentration-response relationship is generated, which is plotted on a graph that depicts a typical S-shaped curve.

The process of determining the LC50 values of a drug based on the response caused in a particular population has been included in areas of special statistical techniques such categorical data analysis. Categorical data employ modeling strategies for describing the relationship between a categorical response variable and a set of explanatory variables. The categorical response variables used in modeling strategies for

describing can be binary, ordinal, nominal, discrete counts or grouped survival times (Stokes et al., 2000). Most often the response of interest for toxicologists is whether or not the concentration of a drug can develop lethal effects on a population. This categorical response is binary because it is constrained to two possible outcomes such as alive or dead, or effect or non-effect for example. Modeling strategies such as Probit and logit have been developed to describe categorical data, and depending on the statistical model the determination of model parameters can be accomplished by the use of maximum likelihood estimation or weighted least squares estimation (Stokes et al., 2000; Rand and Petrocelli, 1984)

Logit analysis or logistic regression analysis is a type of categorical modeling strategy used to predict a response outcome from a set of explanatory variables (predictors). The response variable is usually binary, but it may involve more than two response levels. For toxicological studies the Logistic Regression Model for a binary response variable has been frequently used since the response is bounded to two outcomes of interest, alive and dead, in toxicity trials. The parameters of this model $\propto$ and β (defined below) in the logistic model are estimated with maximum likelihood estimation.

The logistic model involves transforming probabilities into odds. The odds ratio is a measure of association for binary outcomes. It is used to express the likelihood of an event as a proportion of both occurrences and nonoccurrences (Pampel, 2000). For example, the odds for Y=1 is equal to P (Y=1) / [1 – P(Y=1)]. Using logarithmic

transformation of the dependent variable into a nonlinear relationship and considering the logistic distribution as normal distribution, the generalized logistic model can be written

$$\text{Logit}\,(p) = \text{Log}\left[\frac{p_i}{1-p_i}\right] = \propto + \beta x_i$$

Log = Base-10 logarithm of odd ratios
p_i = the probability of an individual in the group is affected by the explanatory variables
$\propto$ = intercept coefficient
β_i = regression coefficient (slope) for the i^{th} predictor
x_i = i^{th} explanatory variable value

Following the logistic model, the concentration levels of a drug at which 50% of the experimental units produce a response (death) can be expressed in terms of probability as:

$$\log = \left[\frac{p_{50}}{1-p_{50}}\right]$$

The logistic parameter estimates from the model are used to compute the log LC50 as follows:

$$\hat{x}_{50} = \frac{-\hat{\propto}}{\hat{\beta}}$$

$\hat{x}_{50}$ = the log LC50 estimate
$\hat{\propto}$ = intercept estimate
$\hat{\beta}$= slope estimate

In order to estimate the actual LC50, the value of $\hat{x}_{50}$ is exponentiated: $\text{LC50} = e^{\hat{x}_{50}}$

The estimate of the variance of LC50 is

$Var(\hat{x}_{50}) = (\hat{x}_{50})^2 \left[\frac{V(\hat{\propto})}{\hat{\propto}^2} - \frac{2V(\hat{\propto},\hat{\beta})}{\hat{\propto}\hat{\beta}} - \frac{V(\hat{\beta})}{\hat{\beta}^2}\right]$ (Adapted from Stokes et al., 2000)

$V(\hat{\propto})$ = the variance of $\hat{\propto}$
$V(\hat{\beta})$ = the variance of $\hat{\beta}$
$V(\hat{\propto},\hat{\beta})$ = the covariance of $\hat{\propto}$ and $\hat{\beta}$

A 100 (1-α) % confidence interval for log LC50 can be computed as

$\hat{x}_{50} \pm = z_{50-\propto/2}\sqrt{var(\hat{x}_{50})}$

Values of $\hat{x}_{50}$ in the confidence limits are also exponentiated for the log scale in order to obtain the actual confidence limits for LC50.

The toxicity of phenol to the brine shrimp *Artemia*, as expressed by LC50 values and their 95% confidence intervals, was estimated by performing logistic regression analysis using the statistical program SAS (version 9.2). LC50 values were calculated after 24h, 48h and 72h individually for test solutions with microspheres and without microspheres. See Table 1.1.

Data from the original proportions used to estimate all the LC50 values were bootstrapped to create 10000 LC50 values which were then compared to compute differences in $\mu_{24h} - \mu_{48h}$; $\mu_{48h} - \mu_{72h}$ and their confidence intervals. In other words, prior to compare LC50 values across time (24h, 48h and 72h) a bootstrap procedure was developed for the unknown population distribution (test organisms) to determine the differences in the proportions as estimated the difference on average and the variability (variances) o these differences. Also, because the intervals were computed with a difference of logs, the logs are transformed into ratios following the properties of logs,

thus all intervals from the testing hypothesis of equal LC50 values are rejected if they do not include 1. See Table 2.1.

To compare test solutions with microspheres and without microspheres the exposure time was fixed (e.g. at 24h, 48h or 72h), then a one-way Analysis of Variances (ANOVA) was conducted. The Fisher's LSD procedure was employed as post-hoc test to determine which LC50 values differ after the null hypothesis of equality of LC50 values was rejected in the analysis of variance. See ANOVA Tables.

The relationship between mortality and test solution concentrations was always sigmoid. See Figs. 5.1-8.1. An analysis of variance was performed to determine if there was a difference in any of the survival percentages between test solutions without microspheres and test solutions with microspheres at each time period. ANOVA was followed by the Fisher's LSD post-hoc procedure to determine which survival percentages among the test solutions were significantly different comparisons with smaller values than 0.05 was judged to be statistically significant. See Table 16.1.

Bootstrap method

Bootstrap is a nonparametric statistical technique that allows inferences about statistical data where conditions of standard parametric methods have been violated (Higgins, 2004). Its applicability includes bootstrap sampling, calculation of standard errors and confidence intervals, analysis of variance, and regression. For the present study, bootstrapping was employed as a statistical approach to estimate the sampling distribution of sample variance from the toxicity tests conducted. Specifically, in this study the original population size for each test solution n=30. A resampling of the data

performing 10000 bootstrap samples was conducted for each pair of proportions as $\mu_{24h}, \mu_{48h}, \mu_{48h}$ and μ_{72h} to obtain their differences as $\mu_{24h} - \mu_{48h}$; $\mu_{48h} - \mu_{72h}$, and their variances across time. The values for the differences between the two population proportions, and their variances (for computing standard errors) were computed as if from random samples from the actual population. The resampling was done with replacement. The bootstrap procedure was performed using computer analysis (SAS version 9.2) and its results are given in Table 2.1.

Finally, an analysis was conducted for the difference of two population means (2-sided) to determine differences among test solutions across time.

CHAPTER TWO

MATERIALS AND METHODS

Importance of this study

The present research examines the impact of microplastics associated with plastic (debris) pollution in the marine environment upon toxicity of an organic compound to a filter-feeding marine organism. Phenol is an organic compound discharged to the environment documented to be toxic to marine and freshwater organisms. The brine shrimp *Artemia* are frequently used as test species for short-terms bioassays of the toxic effects from chemical compounds or natural waters and effluents (Nunes et al., 2005). The susceptibility to pure phenol has been reported in *Artemia* for naupliar stages (Price et al., 1974).

Plastic debris in the marine environment have been pointed out as potential carriers of hydrophobic chemicals and additives of the plastics to the marine biota, presumably increasing the bioavailability of these toxic compounds to the organisms and to their habitats (Teuten et al., 2009; 2007; Mato et al., 2001). However, the potential for microplastics to transport contaminants to a variety of filter-feeding species and enhance toxicity is still limited. In particular, there is a need to investigate other plastics widely used in a large number of applications such as polystyrene to determine its potential as a carrier of organic contaminants to marine organisms as well. More important, the subsequently biological effects in the marine organisms derived from the exposure to the uptake of contaminants from the environment onto microplastics.

Purpose of the study

The overall objective of these experimental trials was to conduct acute toxicity tests for, and determination of, the impact of varying concentrations of plastic microbeads upon the toxicity of a representation organic compound (phenol) to the brine shrimp *Artemia,* used as a surrogate filter-feeding marine organism. The lethal effects of phenol upon *Artemia,* and the interaction with microparticles was examined by;

1) Quantification of the 24h, 48h and 72h LC50 values for phenol in adult *Artemia* populations, at 25 °C, and 30 ppt salinity.
2) Quantification of the 24h, 48h and 72h LC50 values for phenol containing three concentrations of polystyrene microspheres in adult *Artemia* populations at 25 °C, and 30 ppt salinity.

Experimental design

Four acute (24h, 48h and 72h exposure), static non-renewal tests were conducted with the brine shrimp (3-week-old *Artemia*). All brine shrimp nauplii were hatched from commercial cysts available in the market. Nauplii were reared to the adult stage within 3 weeks. Ten brine shrimp of the same size were randomly selected from the *Artemia* culture to conduct the acute toxicity tests. First, the toxicity of phenol alone to the brine shrimp was evaluated. Acute toxicity of phenol as expressed by LC50 values was estimated for the three exposure times. Pure phenol was diluted in synthetic seawater to obtain the test solutions used in trials. The phenol nominal concentrations prepared were 40, 80, 120, 160 and 200 mg L^{-1}. Second, the toxicity of phenol containing microplastic particles to the brine shrimp was evaluated. To accomplish the second part of the study, polystyrene microspheres (3 µm in diameter) were added to phenol. The microsphere concentrations, as expressed by dry weight of spheres, tested were 100, 200 and 300 mg L^{-1}. Toxicity of phenol containing microspheres was also expressed by the LC50 values estimated across time. A series of tests was developed to conduct the present study. Each test was conducted by triplicate, and controls with seawater and brine shrimps were run simultaneously. Tests were considered valid only if mortality in the control did not exceed 10%.

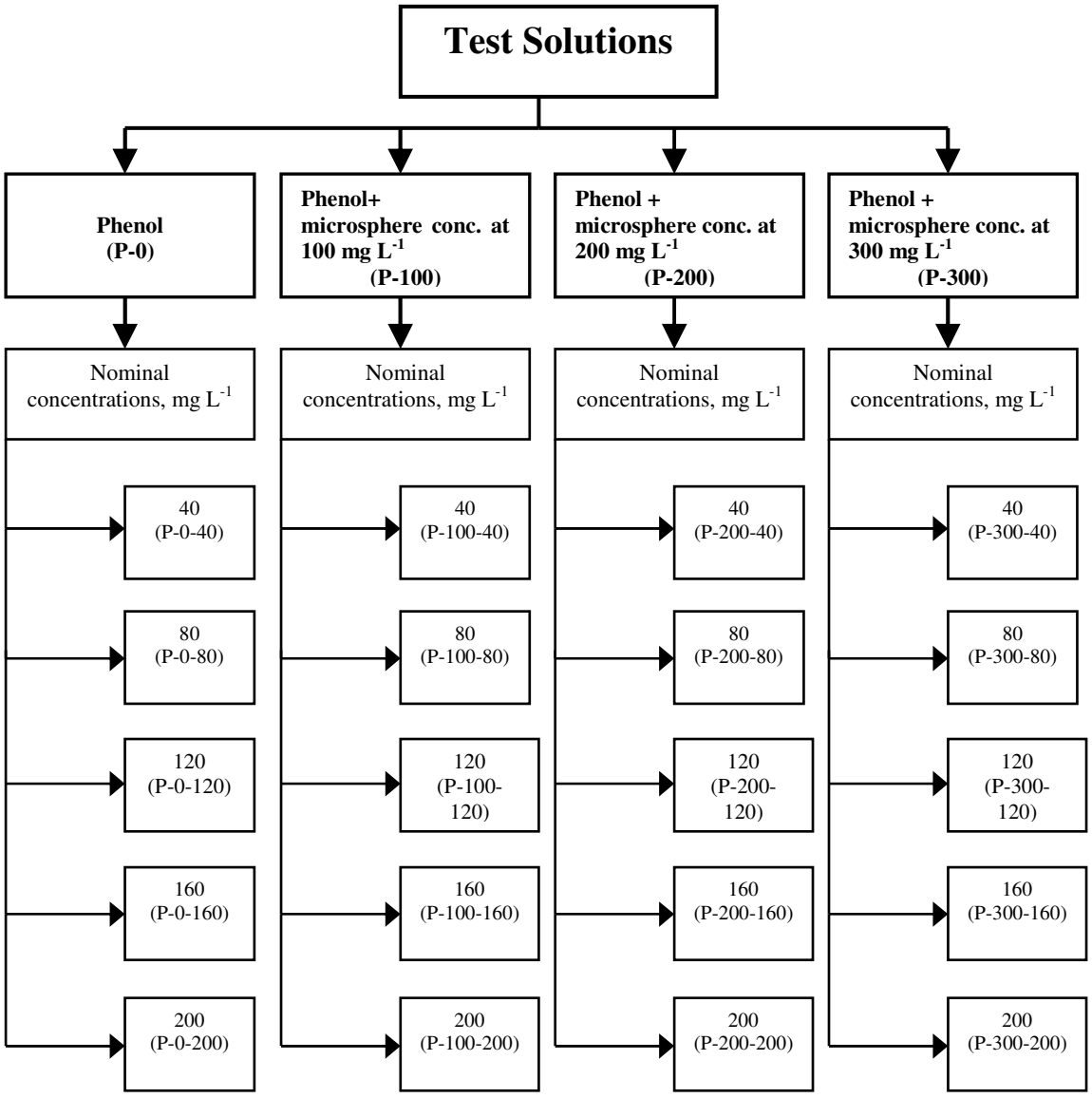


Figure 3.1: Experimental plan graph.

P-0, meaning phenol test solution without microspheres. P-0-40, meaning phenol test solution without microspheres at phenol nominal concentration of 40 mg L^{-1}. Similarly, P-100, meaning phenol test solution with microsphere concentration at 100 mg L^{-1}. P-100-40, meaning phenol test solution with microspheres concentration at 100 mg L^{-1} at phenol nominal concentration of 40 mg L^{-1}.

Brine shrimp *Artemia* culture

Hatching procedures

Premium Grade *Artemia* sp., cysts, obtained from Salt Creek Inc. (Salt Lake City, UT) were hatched adapting the previously reported techniques reported (Sorgeloos et al., 2001; Sorgeloos et al., 1986). For the present study uncapsulated cysts were used to hatch *Artemia* nauplii and all animals were hatched from a single lot. Hatching was accomplished using a 500 mL glass-separatory funnel, a stainless stand and an air system composed of aquarium air pumps, airline and flexible air stones. The sloping sides and stopcock of the glass funnel allowed nauplii to be drained out of the funnel, and to distinguish cyst-hatched nauplii from hatching debris. Separation was based on following light attraction of some naupliar strains reported in aquaculture hatchery practices. The air system equipped with a flexible air stone allowed to aerate from the bottom of the funnel. 1 g of eggs was placed in the funnel filled with synthetic seawater at 30±0.5 ppt salinity and strongly aerated to keep oxygen levels close to saturation and all cysts in suspension. Continuous illumination of about 1000 lux was provided by a fluorescent light tube of 60 W. Water temperature was maintained at 25±0.5 °C and the pH-range was 7.5 to 8.0. Free-swimming nauplii were born after a 24 h incubation period under these technical conditions. For the purpose of this study no hatching percentage was calculated as an indication of egg viability so what was indicated on the commercial can was taken as reference. Finally, upon hatched nauplii were placed into plastic containers with synthetic seawater to complete its larval development.

Adult Artemia culture

Rectangular plastic containers with 3 liters of synthetic seawater were used to grow nauplii. The animal-feeding capability was fully developed after 24 hours. Once the feeding capability was reached food levels were established based on a green algae mixture, *Chlorella sp*. was the single-celled algae dominant. In addition, 1 mL per liter of a commercial liquid food suspension (Florida Aqua Farms Inc., Dade City, FL) was added to containers every day to provide other food. The inoculation with this food mixture was used during a 3-day period. Feed application was just enough to cloud the water so the following application would be when the water was clear. Application was performed at least two times daily. The concentration of algal cells was about $2x10^3$ cells mL^{-1} and monitored with a light microscope and an Improved Neubauer. Identical food concentrations were fed in each growth container. During this period and for the following days, animals were reared in the laboratory using the same apparatus and conditions. Water was continuously aerated by air stones connected with airlines water pumps and the dissolved oxygen was always higher than 7.0 mg L^{-1}. Submersible thermostatic heaters (115V-60 Hz) were used in the water to maintain the temperature at 25°C. Photoperiod, using fluorescent light tubes, was 16h: 8h light: dark. Under these culture conditions, a 3-L container showed more than 500 larvae and produced 250 adults. Water quality was controlled by partial exchange at a daily rate of 25% container volume, complete water renewals every 5 days. Also precipitated material (feces, remained food, dead shrimps) was siphoned out. This procedure prevented to reach ammonia high values.

As larvae matured, individuals were distributed among the containers to achieve a density of 1 adult per 10 mL and minimize crowding. The animals were sampled to determine whether individuals reached adulthood. Adult shrimps were separated and transferred to another container to produce a homogenous population for the experimental trials. No male or female distinction was performed upon separation and only non-pairing individuals were collected. Adults were measured from their head to the abdominal furca using a light microscope provided with an ocular micrometer. Total length for adult individuals without sexual maturity was averaged 8±0.2 mm.

After the 3-day period the live food was replaced gradually for the addition of with food (rice hulls crushed and dried spirulina). The commercial liquid food was kept as routine maintenance feeding in the culture. Animals were fed at least three times per day as the food as consumed. Water in the growth containers was renewed every 3 days during the larval and adult stages to reduce the ammonia.

Test organisms

After 21 days of growing the specimens, starting from the day of hatching of cysts, and before sexual maturity was reached; populations of adult stage organisms were selected randomly to conduct the acute toxicity tests. Animals (average length, 8 mm) were allowed to 24 h of preparation before starting the test. The animals were transferred to containers with clean seawater and fully fed before the test started. Temperature, photoperiod and aeration were kept the same. Salinity values were between 29 and 30 ppt.

Experimental conditions

Phenol concentrations reported are nominal concentrations as the amount of phenol expected to be present in test solutions prepared, while the polystyrene microsphere concentrations are nominal concentrations reported as dry weight of microspheres (mg) present in volume solution (L). In the tests, a group of 10 brine shrimps of the same size were placed in test flasks containing test solutions at phenol nominal concentrations. Controls using only seawater and the brine shrimps were run simultaneously.

A test chamber deigned was composed of 125-mL glass erlenmeyer flasks with perforated screw polyethylene caps and pieces of parafilm used as flask closures, with aeration system and artificial light. The aeration system was developed using 9-in borosilicate glass pipets and 3-in flexible airline tubing connected with a 3-watts/115 V lifter pump (Aquatic Eco-Systems Inc., Apopka, FL), and turned on air. The perforated polyethylene caps (2 mm mean aperture diameter), in each flask, allowed insertion of the tip part of the glass pipets for aeration (Fig. 4.1). A piece of parafilm was stretched across the top of the flask and the cap put on tightly before starting a moderate aeration. Parafilm allowed additional sealing to prevent loss of toxicant content due to the air bubbling. Experience in preliminary tests indicated that a moderate aeration is recommended to maintain microparticles suspended in test solutions. All tests were conducted in an area, at 25 °C, with a 16h: 8h light: dark photoperiod. No feed application was carried on during the tests.

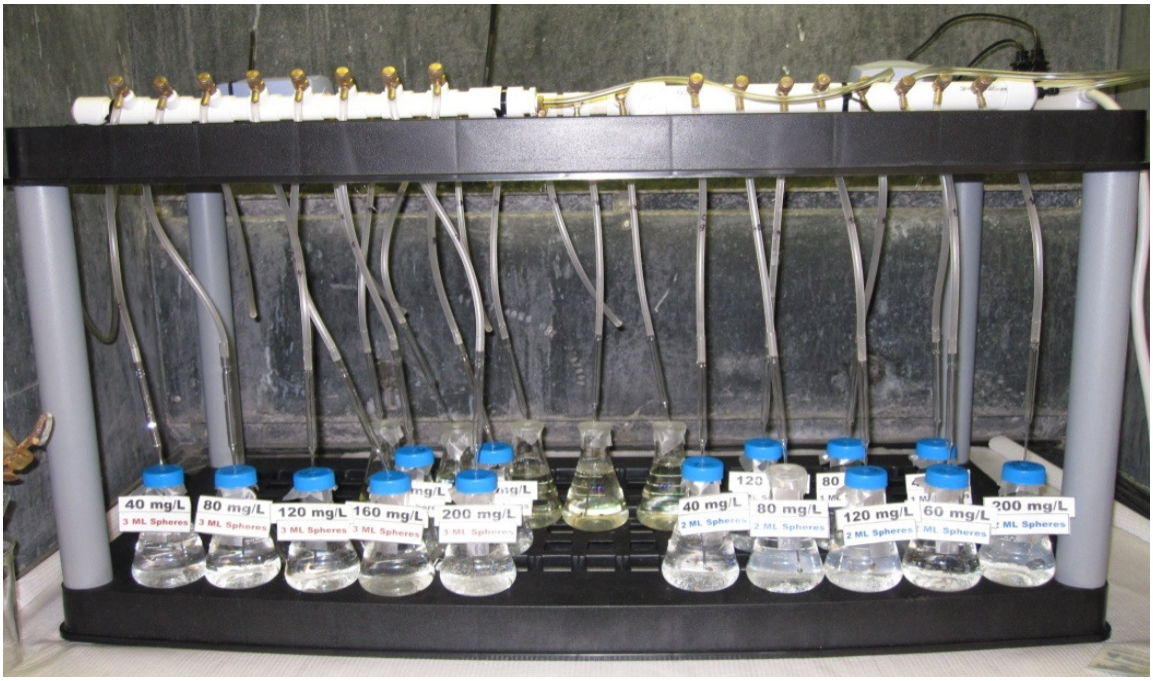


Figure 4.1: Test chamber.

125-mL erlenmeyer flasks, polyethylene taps, cellophane bands, aeration system. Phenol nominal concentrations 40, 80, 120, 160 and 200 mg L^{1} containing microsphere concentrations at 100, 200 and 300 mg L^{-1}.

Dissolved oxygen concentration (DO), pH, salinity, and free ammonia present were determined at the beginning and the end of each test. Dissolved oxygen averaged 8.0 ± 0.5 mg L^{-1}. The pH during the test was observed to be between 7.8 and 8.0. DO was measured with an YSI Model 58 Dissolved-Oxygen Meter (Yellow Springs Instrument Co. Inc., Yellow Springs, OH), and pH was measured with a Thermo Scientific 4 star pH portable meter (Thermo Scientific Inc., Waltham, MA). Free ammonia values were always < 2 mg L^{-1} at the end of test runs, and it was measured with DREL/2010 spectrophotometer (Hatch® Loveland, Colorado).

Synthetic seawater with a salinity of 30 ± 0.5 ppt, prepared in the laboratory, was used as either hatching procedure or culture medium. Synthetic seawater was prepared by mixing commercial salts (Red Sea Salt®) with tap water. To standardize the use of water in the experiment, water was treated with Tap Water conditioner (Fishcare North America Inc., Chalfont, PA) to maximize water quality. The commercial salt was dissolved in pretreated tap water, aerated for 24 h before use in either for culture procedure or test solutions. Synthetic seawater older than 7 days was routinely discarded. The salinity of synthetic seawater at 30 ppt was constant during the experiment.

Phenol concentrations for acute tests

Range-finding test

Toxicity data for the brine shrimp used as organic compounds under laboratory conditions has been documented in the literature (Nunes at el., 2006). Besides Price et al. (1974) reported lethal concentrations for phenol of 157 mg L^{-1} at 24 h, and 56 mg L^{-1} at 48 h using hatched *Artemia* larvae. For the toxicity tests conducted in the present study, a range-finding test was conducted to determine the range of effect-concentrations in the adult *Artemia* populations. The endpoint was mortality at 50% of the organisms recorded after 24h, 48h and 72h. The series of phenol nominal concentrations evaluated were 0, 40, 80, 120, 160 and 200 mg L^{-1}. The lowest concentration with no mortality and the highest concentration with 100 % mortality were chosen as the limits of the definitive test.

Definitive test

The toxicity of phenol alone, as expressed by LC50 values, was determined from the five phenol nominal concentrations investigated. LC50 values were estimated after 24h, 48h and 72h time exposures. Test solution preparation and transfer of the brine shrimp to the solutions did not exceed one hour so potential changes in the toxicant concentration after preparation were reduced. A total of 180 test animals (10 per test flask, 3 replicates) were randomly distributed among test solutions and controls, then kept in the test chamber. The criterion for dead organisms was the inexistence movement on the body for 10 seconds, before dead individuals could be removed from flasks. The brine shrimp being exposed to phenol show a decrease in swimming appendage (flutter) rates and cling together of two shrimps near to death (Price et al., 1974).

Test solutions

Solid phenol (C_6H_5OH) was dissolved directly in the synthetic seawater prepared for culture methods. The chemical reagent-solid was purchased from Sigma-Aldrich Chemical Co. with purity higher than 99%. Phenol was measured using weighing dishes to maintain accuracy on any liquid phenol formation produced by the exposition of the chemical to ambient temperature during weighting. The stock test solution was prepared by dissolving 200 mg of reagent-solid phenol to 1 L of synthetic seawater in 1-L glass bottles. Stocks at 200 mg L^{-1} were used for the preparation of subsequent test solutions. Nominal concentrations of subsequent solutions were made by diluting the stock solution with different volumes of synthetic seawater. Concentrations prepared were 40, 80, 120,

160 and 200 mg L^{-1}, and maintained in tapped 500-mL glass bottles before performing toxicity tests. The procedure was followed during the course of the experiments, and made at the start of each acute test in order to minimize any reduction of phenol concentration in each test solution. Volumetric glass flasks were employed for preparing test solutions to the respective known volume. Each test solution was analyzed for phenol measured concentrations prior to use and was then transferred to 5, the 125-mL glass erlenmeyer flasks. An additional flask was required for the survival control. The volume of each test solution used an all cases was 100 mL. Test glass flasks containing adult brine shrimps were placed in the test chamber under the test conditions previously described.

Analytical chemistry

Water samples from each test solution at the nominal concentrations of 40, 80, 120, 160 and 200 mg L^{-1} were obtained for determination of measured concentrations and to check for any reduction of phenol. The samples were taken from running test solutions and analyzed routinely in the 125-mL Erlenmeyer flasks. Each flask was opened for phenol measurements during the time points of 24h, 48h and 72 h. Phenol concentration in each sample was determined by spectrometric absorption measurements at a detection limit of 1 mg phenol L^{-1} according to the direct photometric method in *Standard Methods* (2005). Samples showing an absorbance band in the wavelength of 500 nm were measured on a spectrophotometer (Milton Roy Spectronic 21D; wavelength range: 340-950 nm; Artisan Scientific Corporation, US), and concentration of phenol determined

using a standard curve generated by measuring phenol of known concentrations of standard solutions. Results of phenol measured concentrations in test solutions are expressed in terms of average measurements and are summarized in APPENDIX B. In general, the percentages of reduction of phenol in test solutions across time were found at 7% (24h), 11% (48h) and 13% (72h) for phenol alone; at 7% (24h), 11% (48h) and 14% (72h) for phenol and microspheres at 100 mg L^{-1}; at 8% (24h); 13% (48h) and 16% (72h) for phenol and microspheres at 200 mg L^{-1}; and at 8% (24h), 15% (48h) and 18% (72h) for phenol and microspheres at 300 mg L^{-1}. The considerations for determining the reduction of phenol, significant differences, in test solutions containing microspheres are discussed in the results and discussion sections.

Phenol and polystyrene microsphere acute tests

The impact of polystyrene spheres (3 µm in diameter, 1% in DI water, Phosphorex®, Fall River, MA) upon the toxicity of phenol to the brine shrimp was examined. Microspheres were in the size range that could be captured by the adult brine shrimp. The concentration levels of polystyrene microspheres studied were 100, 200 and 300 mg L^{-1}. Test solutions at each phenol nominal concentration containing microspheres were evaluated in triplicate. Phenol solutions and their nominal concentrations were prepared following the same procedure when evaluating the toxicity of phenol alone. The experimental conditions such as temperature, salinity, artificial light and aeration were also maintained.

The addition of the polystyrene microspheres was performed soon after each the 125-mL test flask was filled to 100 mL with test solution. Microsphere volumes were transferred using a 1-µm automatic pipette from the microsphere suspension bottle (1 L, Phosphorex®, Fall River, MA) into each test flask. Microspheres were mixed in the solutions by moderate aeration during 15 minutes. During the exposure times aeration was maintained to accomplish particles remained suspended. Appropriate volumes of microsphere suspension were used in each test solution to provide final particle concentrations of 100, 200 and 300 mg L^{-1}. Disposable tips were used when operating the micropipette. Test solutions containing microspheres were properly labeled (Fig. 4.1).

Groups of 10 brine shrimp of the same size were placed, using a plastic Pasteur pipette, into each test solution containing microspheres. This procedure was performed each microsphere concentration level. Controls with seawater and brine shrimps were run simultaneously. The criterion for dead organisms was the same as that used for the phenol test run. No feeding application was added to test solutions containing microspheres. Small aliquots of test solutions containing microspheres were taken for phenol measurements and determination of reduction of phenol concentrations after 24h, 48h and 72h. The phenol measurements obtained were used to calculate the LC50 values and their confidence intervals.

Dry weight determination

Four runs were conducted to quantify the microsphere uptake of adult *Artemia* kept in synthetic saltwater media (30 ppt salinity, and 25°C), as function of particle concentration and time. Runs were: S-0 (seawater without microparticles); S-100 (seawater with micropartcles at 100 mg L^{-1}); S-200 (seawater with microparticles at 200 mg L^{-1}); S-300 (sweater with microparticles at 300 mg L^{-1}). The brine shrimp were kept in 120-mL glass flasks, filled with 100 mL of seawater and moderate aeration, and artificial light. The three microsphere concentrations (100, 200 and 300 mg L^{-1}) were individually added to the flasks. Mixing was accomplished by aeration for 15 minutes prior the brine shrimp were put in flasks. Samples of 5 animals were weighted after 24-h, 48-h and 72-h, using Cahn Model 26 automatic electrobalance to determine the individual brine shrimp dry weight from S-0, S-100, S-200 and S-300 runs.

The brine shrimp in the samples were washed with ammonium formate solution (0.5 Molar) over a sieve (10 cm in diameter) to remove feces. Animals were then filtered onto predried, tared glass microfiber filters, then dried and weighed. The filter size was 25 mm for the adult brine shrimp. In this way, individual weight was determined within plus or minus 0.2. See Table APPENDIX C.

Results

Table 1.1: Estimated LC50 values using logistic analysis for test solutions.

Microsphere concentrations as 100 mg L^{-1} (C1); 200 mg L^{-1} (C2); 300 mg^{-1} (C3).

24-h measured estimated phenol LC50		
Test Solution	**24-h LC50 (mg L^{-1})**	**95% C.I.**
Phenol alone	211.06	157.60; 285.23
Phenol + C1	213.33	161.59; 281.65
Phenol + C2	213.72	158.76; 287.66
Phenol + C3	216.41	160.66; 291.45
48-h measured estimated phenol LC50		
Test Solution	**48-h LC50 (mg L^{-1})**	**95% C.I.**
Phenol alone	113.19	103.18; 124.17
Phenol + C1	106.45	103.42; 109.57
Phenol + C2	121.34	110.44; 133.28
Phenol + C3	175.15	136.70; 224.43
72-h measured estimated phenol LC50		
Test Solution	**72-h LC50 (mg L^{-1})**	**95% C.I.**
Phenol alone	90.90	83.22; 99.29
Phenol + C1	91.57	83.81; 100.04
Phenol + C2	100.25	91.58; 109.71
Phenol + C3	116.75	106.34; 128.13

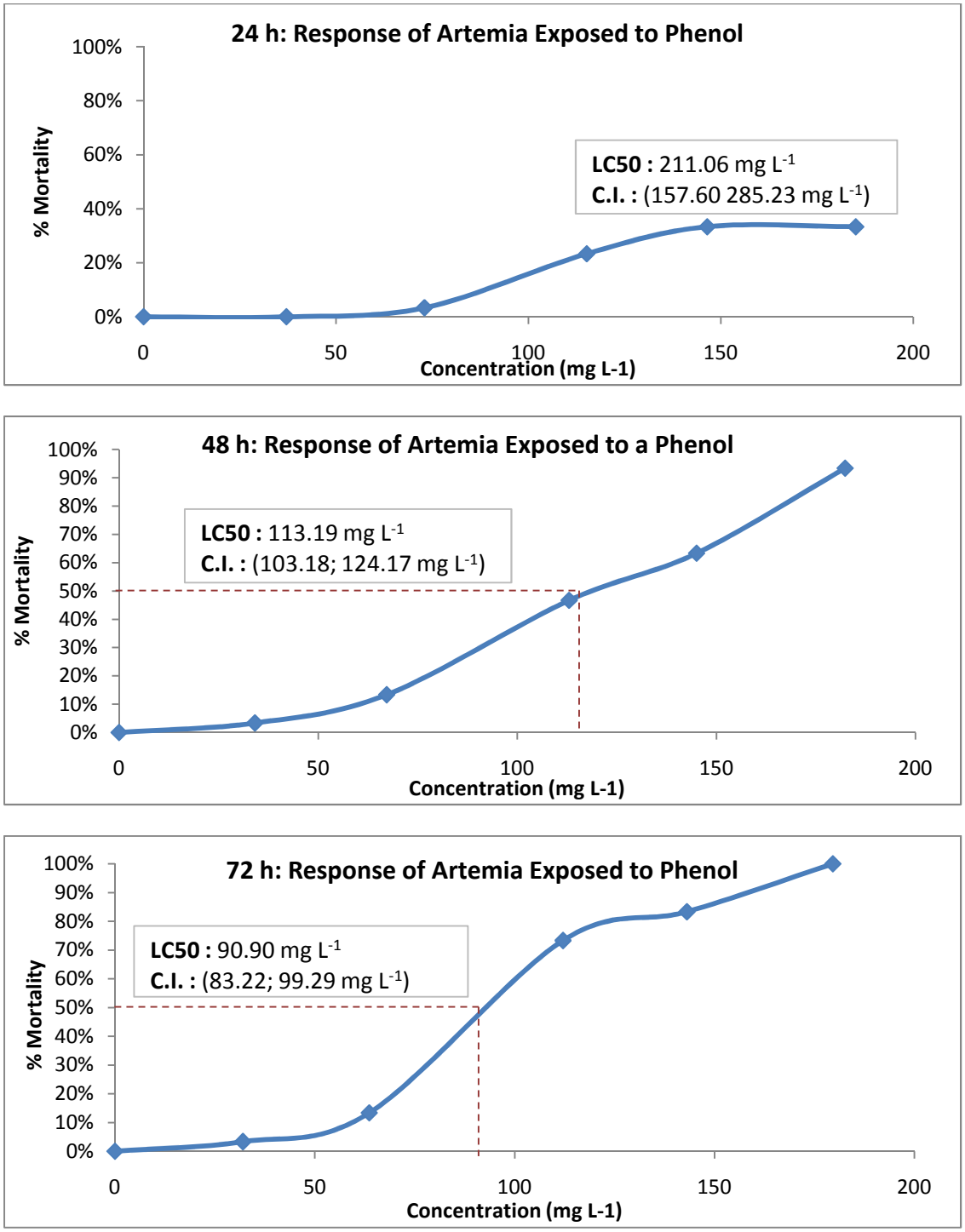
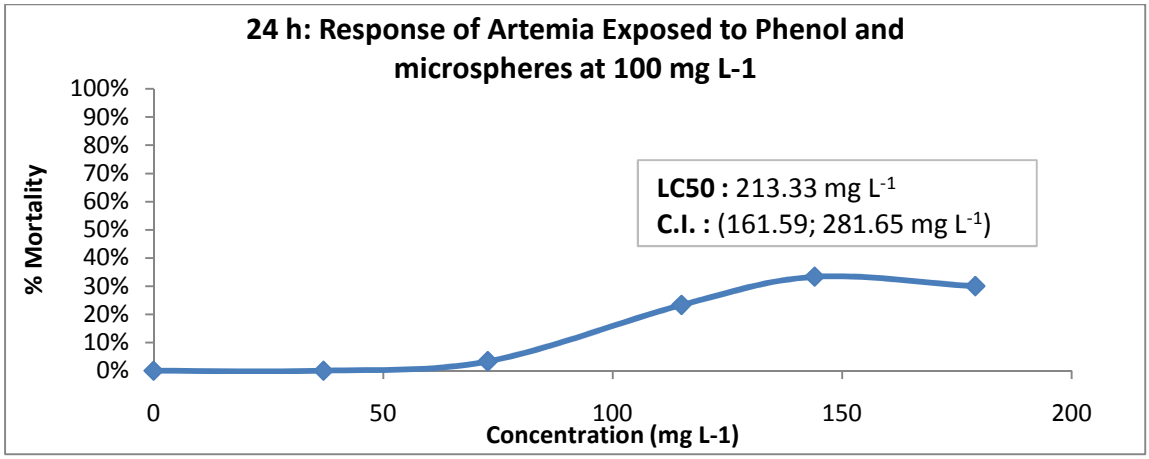
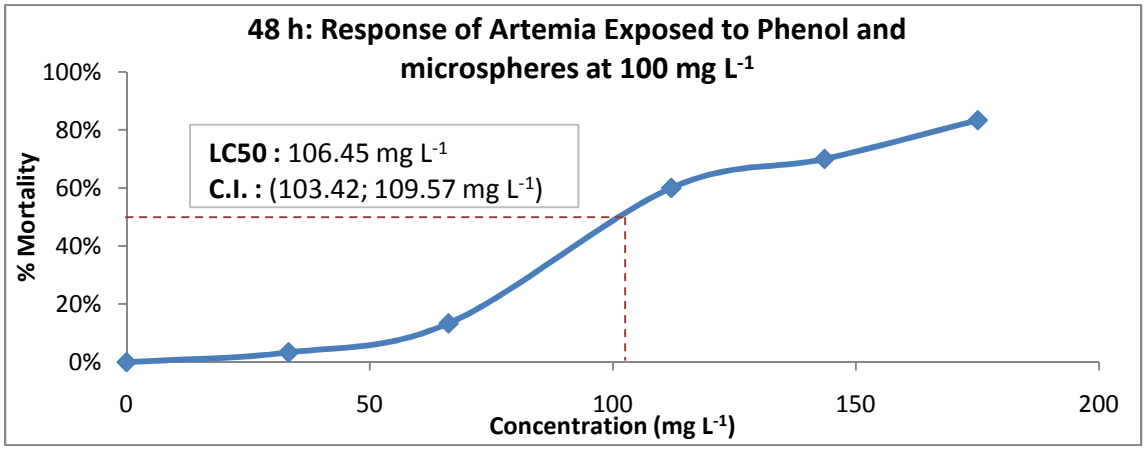


Figure 5.1: Estimated toxicity curves across time for phenol alone using logistic analysis.

Lethal concentrations (mg L^{-1}) to 50% of the *Artemia* (LC50) for each exposure time, with confidence intervals (C.I.) are indicated.





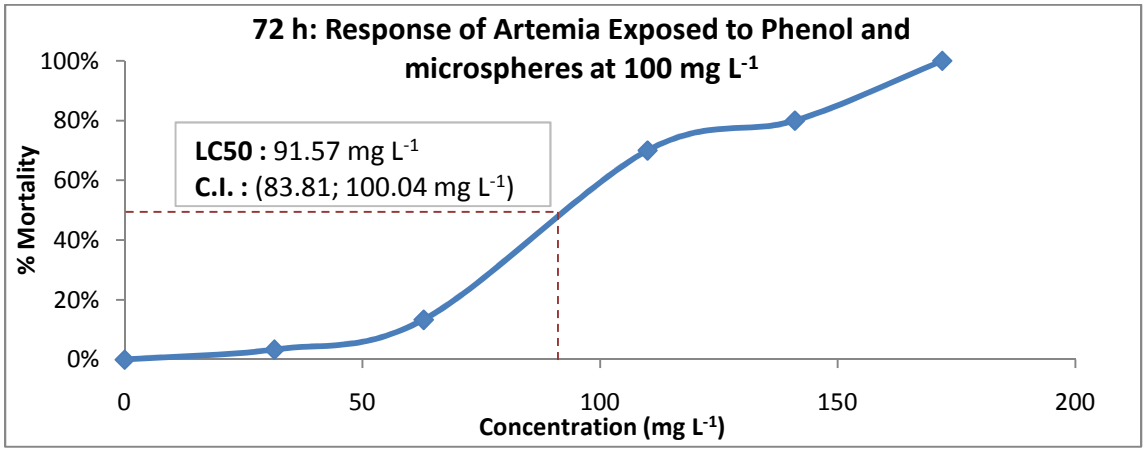
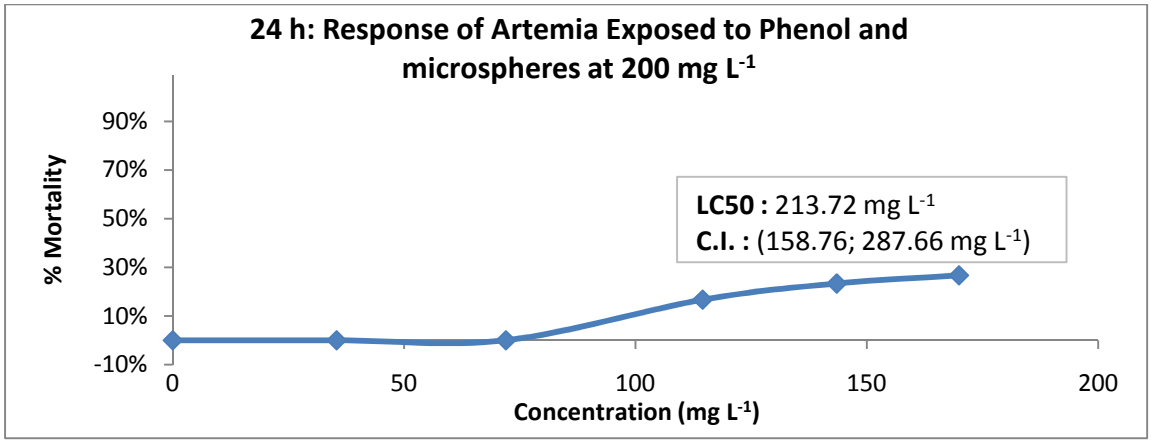
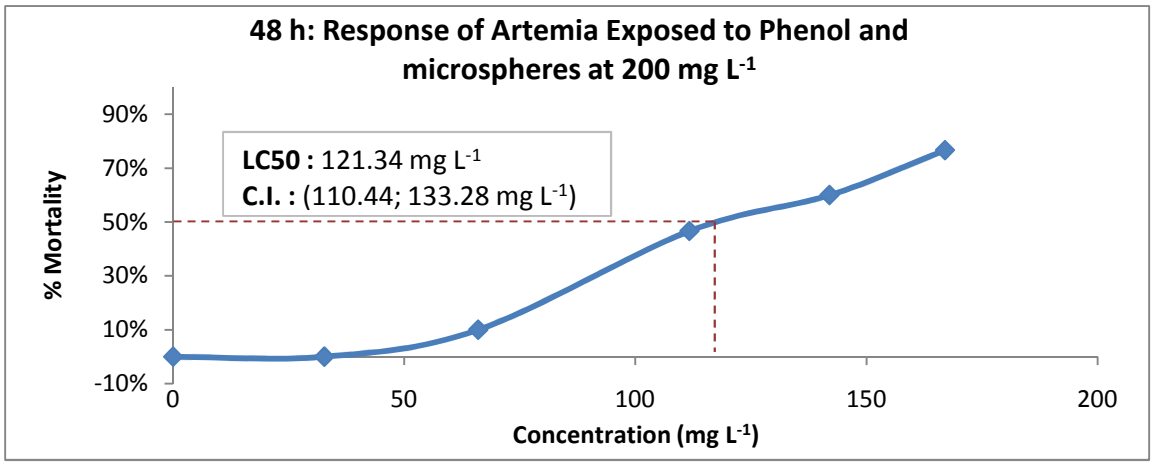


Figure 6.1: Estimated toxicity curves for phenol and microspheres at 100 mg L^{-1} across time using logistic analysis.

Lethal concentrations (mg L^{-1}) to 50% of the *Artemia* (LC50) for each exposure time, with confidence intervals (C.I.) are indicated.





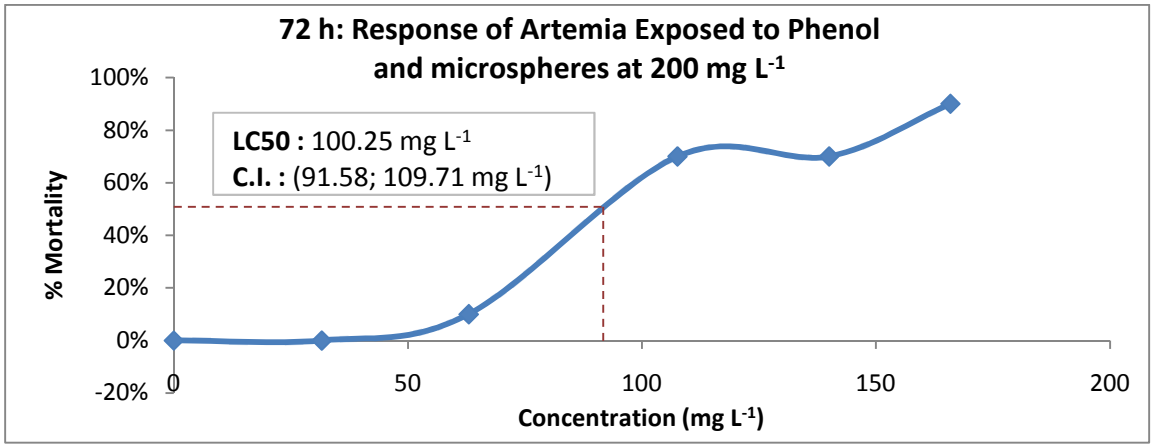


Figure 7.1: Estimated toxicity curves for phenol and microspheres at 200 mg L^{-1} across time using logistic analysis.

Lethal concentrations (mg L^{-1}) to 50% of the *Artemia* (LC50) for each exposure time, with confidence intervals (C.I.) are indicated.

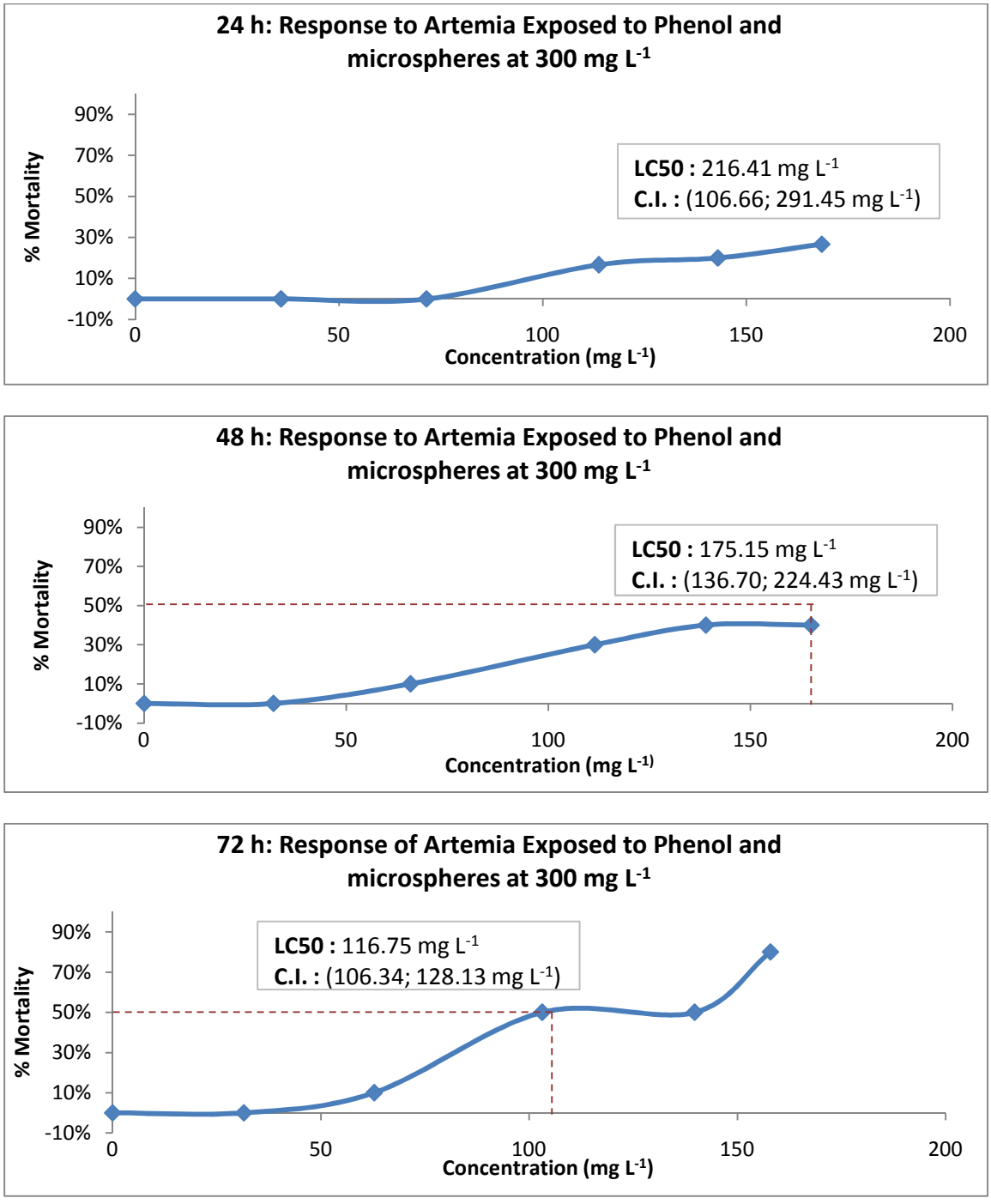


Figure 8.1: Estimated toxicity curves for phenol and microspheres at 300 mg L^{-1} across time using logistic analysis.

Lethal concentrations (mg L^{-1}) to 50% of the *Artemia* (LC50) for each exposure time, with confidence intervals (C.I.) are indicated.

Analysis for comparing the differences in LC50 values using bootstrap samples

Table 2.1: Bootstrap table for the differences in LC50 values analysis.

95% Confidence Interval (C.I.).

1000 bootstrap samples				
Test Solution	$\mu_{24h} - \mu_{48h}$	**C.I.**	$\mu_{48h} - \mu_{72h}$	**C.I.**
Phenol alone	1.87	(0.95, 3.63)	1.25	(0.91, 1.70)
Phenol+C1	2.0	(0.95, 4.22)	1.16	(0.85, 1.59)
Phenol+C1	1.77	(0.90, 3.40)	1.21	(0.92, 1.58)
Phenol+C2	1.23	(0.45, 3.41)	1.51	(0.73, 3.11)

Using the data summarized in Table 2.1 an analysis for comparing the differences in the LC50 values from μ_{24h} and μ_{48h}, μ_{48h} and μ_{72h} was performed, as follows:

$\mu_{24h} - \mu_{48h}$ = difference between 24h-LC50 and 48h-LC50 values

$\mu_{48h} - \mu_{72h}$= difference between 48h-LC50 and 72h-LC50 values

Hypotheses: H_o: $\mu_{24h} = \mu_{48h}$

H_a: $\mu_{24h} \neq \mu_{48h}$

Hypotheses: H_o: $\mu_{48h} = \mu_{72h}$

H_a: $\mu_{48h} \neq \mu_{72h}$

These hypotheses were used to test the difference between two LC50 values for the test solutions with microspheres and without microspheres across time (24h, 48h and 72h). The decisions to reject or fail to reject the null hypothesis are based on the 95% confidence interval (C.I.) for each differences found. If the C.I. include do not include 1, the null hypothesis is rejected.

Results from Table 2.1 indicated no significant differences were found in the LC50 values in the four test solutions analyzed for the differences between μ_{24h} and μ_{48h}. Since the computed 95% C.I. from comparing μ_{24h} and μ_{48h} includes 1, for the all the test solutions, the null hypothesis of no different between the 24h-LC50 and 48h-LC50 ($\mu_{24h} - \mu_{48h}$) was not rejected. Therefore, there was not statistical evidence that the 24h-LC50 values were different than the 48h-LC50 values for the following test solutions: phenol alone as its 24h-LC50 was 211.06 mg L^{-1} and a 48h-LC50 of 113.19 mg L^{-1}; phenol with microspheres at 100 mg L^{-1} as its 24h-LC50 was 213.33 mg L^{-1} and a 48h-LC50 of 106.45 mg L^{-1}; and phenol with microspheres at 200 mg L^{-1} as its 24h-LC50 was 213.72 mg L^{-1} and a 48h-LC50 of 121.34 mg L^{-1}. When comparing the LC50 values between μ_{48h} and μ_{72h} no difference in the 48h-LC50 and 72h-LC50 values was observed for test solutions independent of the presence of microspheres.

One-way analysis of variance to compare LC50 values across test solutions

Table 3.1: ANOVA table for analysis of 24h-LC50 values.

Source of variance	**Sum of squares**	**Degree of freedom**	**Mean Squares**	**F test**
Treatment	43.2942	3	14.4314	647.51
Error	0.1783	8	0.0223	
Totals	43.4725	11		

Hypotheses: H_o: $\mu_0 = \mu_1 = \mu_2 = \mu_3$

H_a: At least one of the μ differs from the rest

μ_0 = average 24h-LC50 value for phenol alone
μ_1 = average 24h-LC50 value for phenol and microsphere concentration at 100 mg L^{-1}
μ_2 = average 24h-LC50 value for phenol and microsphere concentration at 200 mg L^{-1}
μ_3 = average 24h-LC50 value for phenol and microsphere concentration at 300 mg L-1

The critical value for $F_{0.05,3,8} = 4.07$. Because the computed value of the test statistic (F), 647.51 exceeds the critical value of F, 4.07, the null hypothesis (H_o) of equality of the average 24h-LC50 values was rejected. To investigate if the 24h-LC50 values from test solutions containing microsphere concentrations were significantly greater than the 24h-LC50 values from the test solution without microspheres, all pairwise comparison procedure (Fisher's LSD) was conducted.

$$LSD = t_{\propto/2,8}\sqrt{\frac{2\,MSE}{n}}$$

$$LSD = 2.306\sqrt{2\left(\frac{0.0223}{11}\right)} = 0.1468$$

Table 4.1: Pairwise comparison of the 24h-LC50 values.

Microspheres concentrations: C1: 100 mg L^{-1}; C2=200 mg L^{-1}; C3=300 mg L^{-1}.

Comparison	LC50 differences	LSD	Decision
72h-LC50 (Phenol+C3) – 72h-LC50 (Phenol alone)	5.35	> 0.1468	A pairwise difference exists
72h-LC50 (Phenol+C2) – 72h-LC50 (Phenol alone)	2.66	> 0.1468	A pairwise difference exists
72h-LC50 (Phenol+C1) – 72h-LC50 (Phenol alone)	2.27	> 0.0451	A pairwise difference exists
72h-LC50 (Phenol+C3) – 72h-LC50 (Phenol+C2)	2.69	> 0.0451	A pairwise difference exists
72h-LC50 (Phenol+C3) – 72h-LC50 (Phenol+C1)	3.04	> 0.0451	A pairwise difference exists
72h-LC50 (Phenol+C2) – 72h-LC50 (Phenol+C1)	0.39	> 0.0451	A pairwise difference exists

Results from all pairwise comparisons in Table 4.1 indicated that the 24h-LC50 values were significantly different from each other at 24h. In this case the 24h-LC50 values from test solutions containing microspheres at the three microsphere concentrations as 213.33 mg L^{-1} (C1); 213.72 mg L^{-1} (C2) and 216.41 mg L^{-1} (C3) are higher than the 24h-LC50 of 211.06 mg L^{-1} from phenol alone. Moreover, the differences

among the 24h-LC50 values involving test solutions with microspheres were statistically significant.

Table 5.1:. ANOVA table for analysis of 48h-LC50 values.

Source of variance	Sum of squares	Degree of freedom	Mean Squares	F test
Treatment	8840.83	3	2946.94	568084.81
Error	0.0415	8	5.1875×10^{-3}	
Totals	8840.87	11		

Hypotheses: H_o: $\mu_0 = \mu_1 = \mu_2 = \mu_3$

H_a: At least one of the μ differs from the rest

μ_0 = average 48h-LC50 value for phenol alone
μ_1 = average 48h-LC50 value for phenol and microsphere concentration at 100 mg L^{-1}
μ_2 = average 48h-LC50 value for phenol and microsphere concentration at 200 mg L^{-1}
μ_3 = average 48h-LC50 value for phenol and microsphere concentration at 300 mg L^{-1}

The critical value for $F_{0.05,3,8} = 4.07$. Table 5.1 shows the computed value of the test statistic (F), 568084.81 exceeded the critical value of F, 4.07, thus the null hypothesis of equality of the 48h-LC50 values was rejected. As for the 24h-LC50 values, the Fisher's LSD comparison procedure was conducted to determine differences in the 48h-LC50 values from test solutions with microspheres and without microspheres.

$$LSD = 2.306\sqrt{2\left(\frac{2.1875\text{x}10^{-3}}{11}\right)} = 0.0459$$

Table 6.1: Pairwise comparison of the 48h-LC50 values.

Comparison	LC50 differences	LSD	Decision
72h-LC50 (Phenol+C3) – 72h-LC50 (Phenol alone)	61.96	> 0.0459	A pairwise difference exists
72h-LC50 (Phenol+C2) – 72h-LC50 (Phenol alone)	8.15	> 0.0459	A pairwise difference exists
72h-LC50 (Phenol+C1) – 72h-LC50 (Phenol alone)	$\lvert -6.74 \rvert$	> 0.0459	A pairwise difference exists
72h-LC50 (Phenol+C3) – 72h-LC50 (Phenol+C2)	53.81	> 0.0459	A pairwise difference exists
72h-LC50 (Phenol+C3) – 72h-LC50 (Phenol+C1)	68.70	> 0.0459	A pairwise difference exists
72h-LC50 (Phenol+C2) – 72h-LC50 (Phenol+C1)	14.89	> 0.0459	A pairwise difference exists

In doing all pairwise comparisons of 48h-LC50 values in Table 6.1 indicated that the 48h-LC50 values were significantly different from each other at 48h. The 48h-LC50 values from test solutions with microspheres as 175.15 mg L^{-1} (C3), 121.34 mg L^{-1} (C2) and 106.45 mg L^{-1} (C1) are higher than the 48h-LC50 of 113.19 mg L^{-1} for phenol alone. Likewise, the 48h-LC50 values among the test solutions containing microspheres were found significant differences as well.

Table 7.1: ANOVA table for analysis of 72h-LC50 values.

Source of variance	Sum of squares	Degree of freedom	Mean Squares	F test
Treatment	6092.47	3	2030.8233	961336.4734
Error	0.0169	8	2.1125×10^{-3}	
Totals	6092.48	11		

Hypotheses: H_o: $\mu_0 = \mu_1 = \mu_2 = \mu_3$

H_a: At least one of the μ differs from the rest

μ_0 = average 72h-LC50 value for phenol alone
μ_1 = average 72h-LC50 value for phenol and microsphere concentration at 100 mg L^{-1}
μ_2 = average 72h-LC50 value for phenol and microsphere concentration at 200 mg L^{-1}
μ_3 = average 72h-LC50 value for phenol and microsphere concentration at 300 mg L^{-1}

The critical value for $F_{0.05,3,8} = 4.07$. The ANOVA table 7.1 shows that the computed value of the test statistic (F), 961336.4734 exceeded the critical value of F, 4.07, thus the null hypothesis of equality of the 72h-LC50 values was rejected. To determine whether all 72h-LC50 values from test solutions with microspheres are significantly greater than the 72h-LC50 value from test solution without microspheres, a multiple comparison procedure was conducted.

$$LSD = 2.306\sqrt{2\left(\frac{2.1125\text{x}10^{-3}}{11}\right)} = 0.0451$$

Table 8.1: Pairwise comparison of the 72h-LC50 values.

Comparison	LC50 differences	LSD	Decision
72h-LC50 (Phenol+C3) – 72h-LC50 (Phenol alone)	25.85	> 0.0451	A pairwise difference exists
72h-LC50 (Phenol+C2) – 72h-LC50 (Phenol alone)	9.35	> 0.0451	A pairwise difference exists
72h-LC50 (Phenol+C1) – 72h-LC50 (Phenol alone)	0.67	> 0.0451	A pairwise difference exists
72h-LC50 (Phenol+C3) – 72h-LC50 (Phenol+C2)	16.5	> 0.0451	A pairwise difference exists
72h-LC50 (Phenol+C3) – 72h-LC50 (Phenol+C1)	25.18	> 0.0451	A pairwise difference exists
72h-LC50 (Phenol+C2) – 72h-LC50 (Phenol+C1)	8.68	> 0.0451	A pairwise difference exists

Determining pairwise comparisons among the 72h-LC50 values in Table 8.1 showed that significant differences were observed. The corresponding 72h-LC50 values for test solutions with microspheres for 116.75 mg L^{-1} at C3; 100.25 mg L^{-1} at C2; and 91.57 mg L^{-1} at C1 were higher than the 72h-LC50 of 90.90 mg L^{-1} for phenol alone. Also, significant differences were observed among test solutions with microspheres by performing further comparisons.

Survival percentage comparisons

Data from the survival percentages observed in test solutions at different concentrations of phenol and microspheres were examined using one-way ANOVA and multiple comparison procedures to determine difference in survivals between solutions with microspheres and without microspheres. The ANOVA tables and summary of the multiple comparisons for data with significant differences in survival percentages are shown in Tables 9.1; 10.1; 11.1; 12.1; 13.1

For the group of test solutions at nominal concentrations of 120 mg L^{-1}, a significant difference in the survival was found at 48 h and 72 h (F_{obs} =60.0, *P*-value 0.0002 and F_{obs}=41.0, *P*<0.0001, respectively). The survival observed in P-200-120 and P-300-120 at 48 h, and in P-300-120 at 72 h was higher than the survival for P-0-160. See Fig. 11.1

For the group of test solutions at nominal concentrations of 160 mg L^{-1}, significant differences in the survival were observed at 24 h, 48 h and 72 h (F_{obs}=0.0222, *P*-values 0.0222; F_{obs}=60.0, 0.0002; F_{obs}=81.0, *P*<0.0001, respectively). The survival percentage observed in P-200-160 and P-300-160 was higher than P-0-160 at 24 h, while at 48h the survival was only high in P-300-160, and at 72h the survival was found higher than P-0-160 in P-200-160 and P-300-160. See Fig. 12.1

For the group of test solutions at nominal concentrations at 200 mg L^{-1}, significant difference in the survival percentages were observed only at 48 h and 72 h (F_{obs}=64.89, *P*-values <0.0001 and F_{obs}=*Infty*, <0.0001, respectively). The survival in P-200-200 and

P-300-200 was higher than P-0-200 at 48 h. Finally, the survival was found higher than P-0-200 in P-200-200 and P-300-200. See Fig. 13.1

Table 9.1: ANOVA table for analysis of survival at 120 mg L^{-1} of phenol at 48h.

Source of variance	**Sum of squares**	**Degree of freedom**	**Mean squares**	**F test**	***p*-value**
Treatment	1358.3333	3	452.7778	27.17	0.0002
Error	133.3333	8	16.6667		
Totals	1491.6667	11			

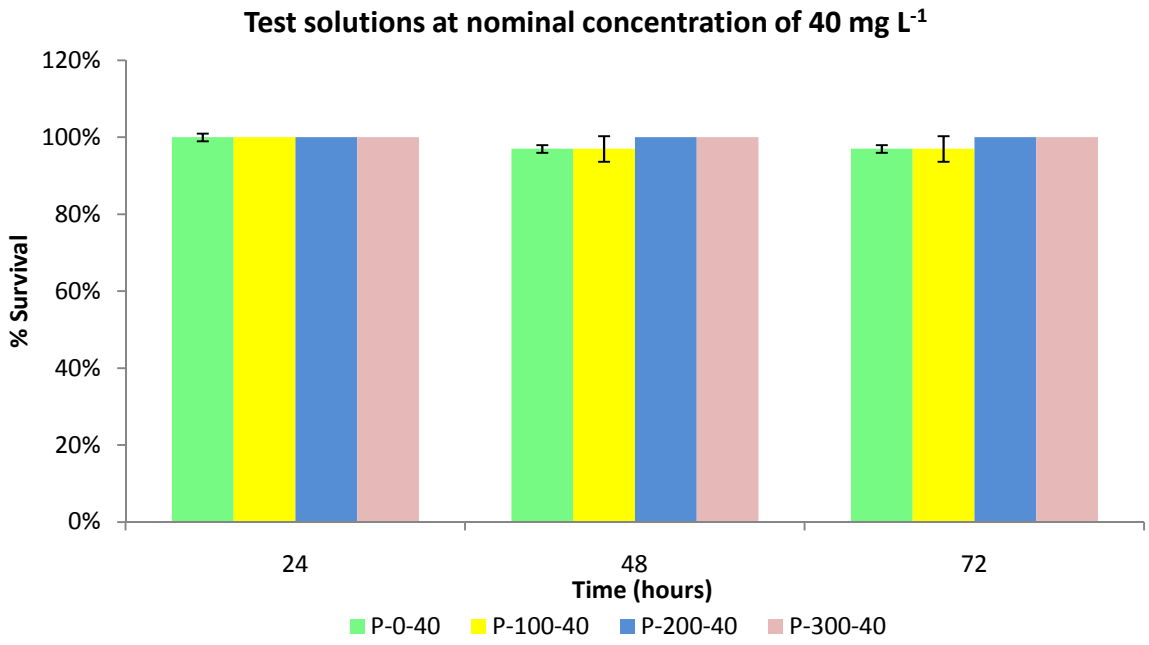


Figure 9.1: Survival percentages of adult *Artemia* exposed to phenol at 40 mg L^{-1} containing microspheres as function of time.

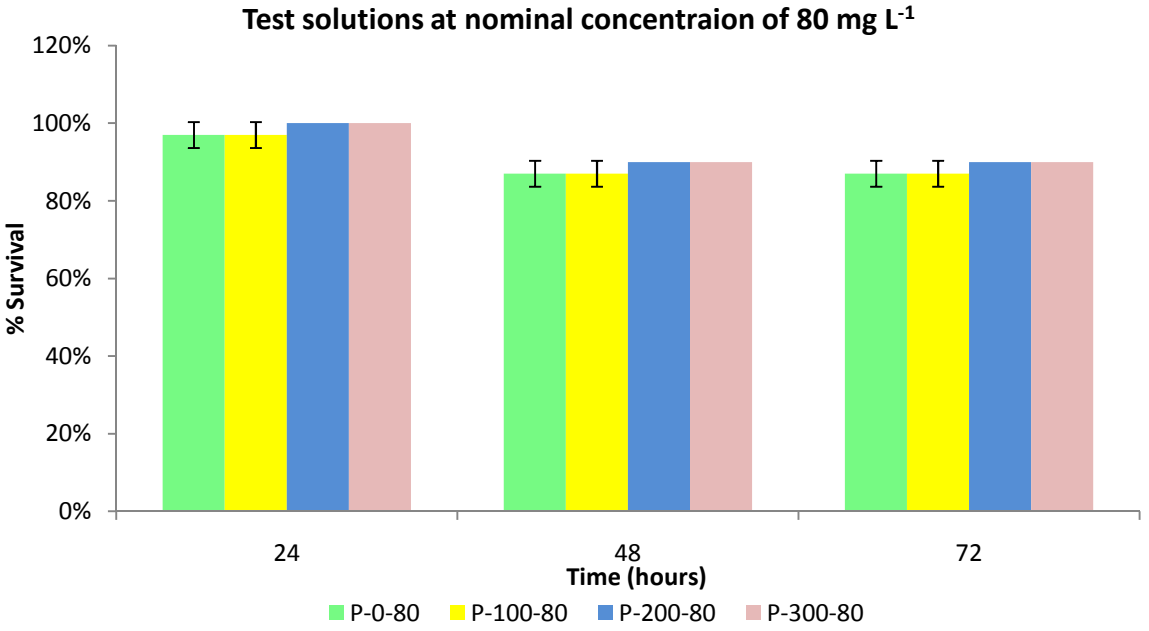


Figure 10.1: Survival percentages of adult *Artemia* exposed to phenol at 80 mg L^{-1} containing microspheres as function of time.

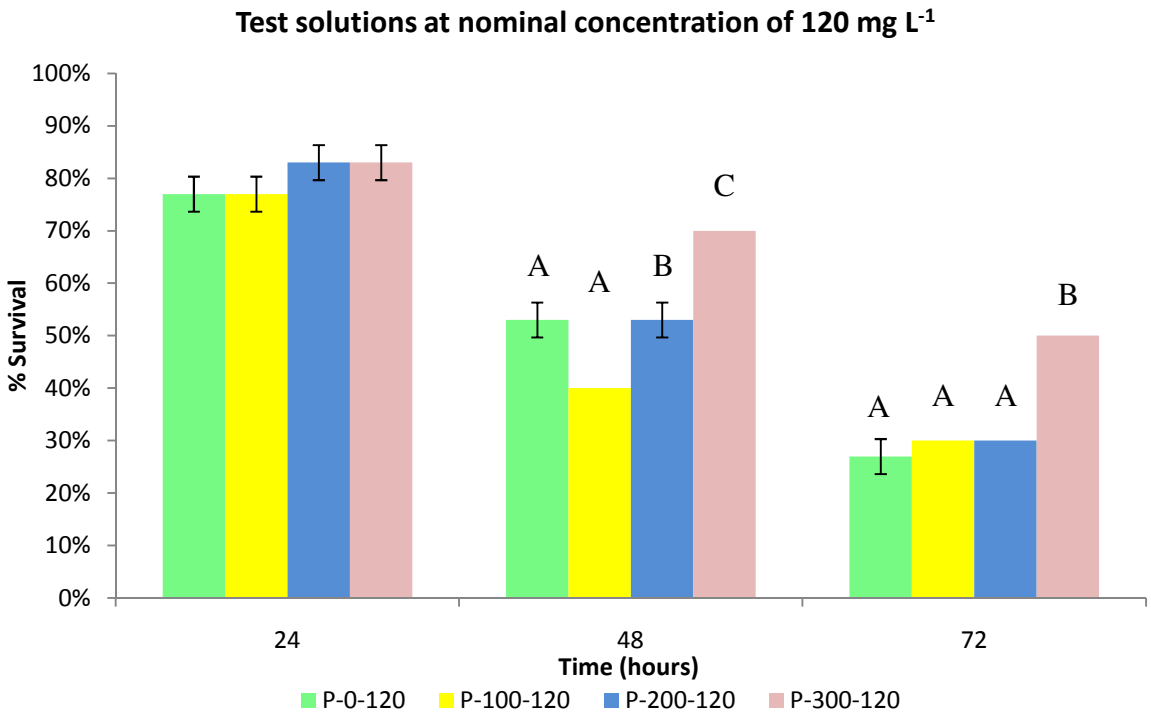


Figure 11.1: Survival percentages of adult *Artemia* exposed to phenol at 120 mg L^{-1} containing microspheres as function of time.

Mean value of three replicate experiments for each concentration ± S.E (bars). The solution concentration where significant differences in survival were found was marked with a different letter.

Table 10.1: ANOVA table for analysis of survival at 120 mg L^{-1} of phenol at 72h.

Source of variance	**Sum of squares**	**Degree of freedom**	**Mean squares**	**F test**	***p*-value**
Treatment	1025.0	3	341.6667	41.0	< .0001
Error	66.6667	8	8.3333		
Totals	1091.6667	11			

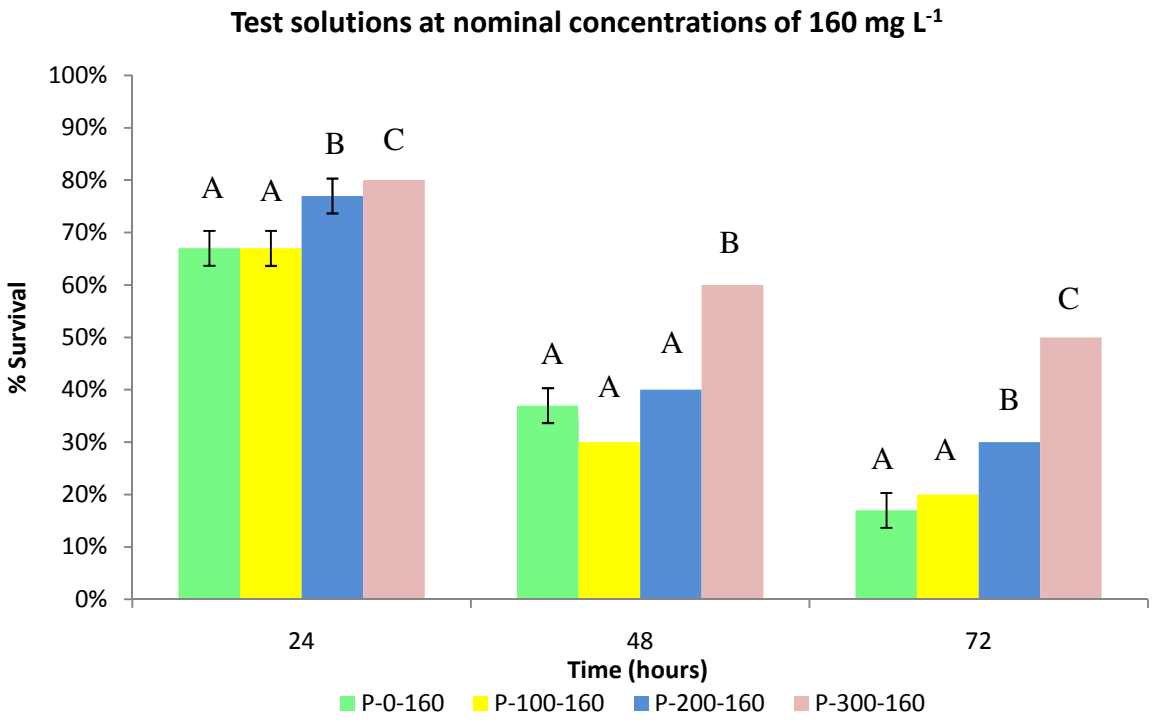


Figure 12.1: Survival percentages of adult *Artemia* exposed to phenol at 160 mg L^{-1} containing microspheres as function of time.

Mean value of three replicate experiments for each concentration ± S.E (bars). The solution concentration where significant differences in survival were found was marked with a different letter.

Table 11.1: ANOVA table for analysis of survival at 160 mg L^{-1} of phenol at 24h.

Source of variance	Sum of squares	Degree of freedom	Mean squares	F test	*p*-value
Treatment	425.0	3	141.6667	5.67	0.0222
Error	200.0	8	25.0		
Totals	625.0	11			

Table 12.1: ANOVA table for analysis of survival at 160 mg L^{-1} of phenol at 48h.

Source of variance	Sum of squares	Degree of freedom	Mean squares	F test	*p*-value
Treatment	1500.0	3	500.0	60.0	< .0001
Error	66.6667	8	8.3333		
Totals	1566.6667	11			

Table 13.1: ANOVA table for analysis of survival at 160 mg L^{-1} of phenol at 72h.

Source of variance	Sum of squares	Degree of freedom	Mean squares	F test	*p*-value
Treatment	2025.0	3	675.0	81.0	< .0001
Error	66.6667	8	8.3333		
Totals	2091.6667	11			

Table 14.1: ANOVA table for analysis of survival at 200 mg L^{-1} of phenol at 48h.

Source of variance	Sum of squares	Degree of freedom	Mean squares	F test	*p*-value
Treatment	4866.6667	3	1622.2222	64.89	< .0001
Error	200.0	8	25.0		
Totals	5066.6667	11			

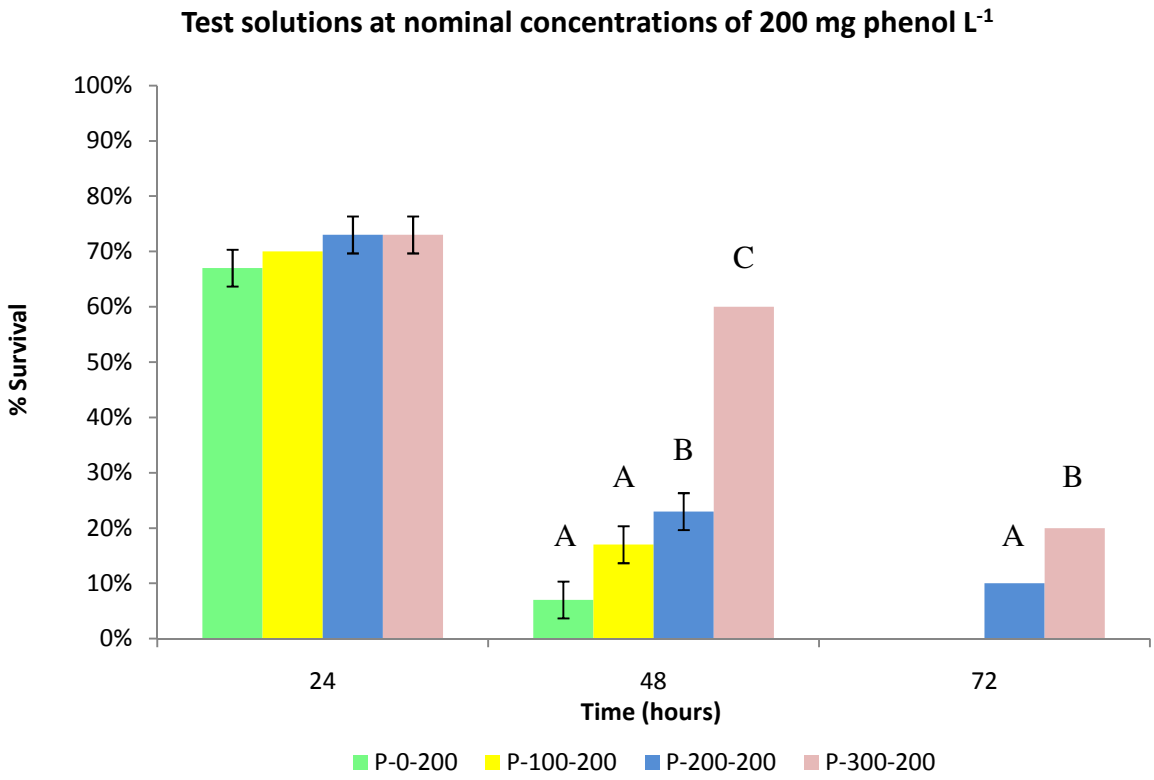


Figure 13.1: Survival percentages of adult *Artemia* exposed to phenol at 200 mg L^{-1} containing microspheres as function of time.

Mean value of three replicate experiments for each concentration ± S.E (bars). The solution concentration where significant differences in survival were found was marked with a different letter.

Table 15.1: ANOVA table for analysis of survival at 200 mg L^{-1} of phenol at 72h.

Source of variance	Sum of squares	Degree of freedom	Mean squares	F test	*p*-value
Treatment	825.0	3	275.0	Infty	< .0001
Error	0	8	0		
Totals	825.0	11			

Reduction of phenol from test solutions

Throughout all experimental trials phenol concentration was determined in all test solutions without spheres and with spheres to determine the amount of phenol potentially lost to degradation or volatilization. See APENDIX B. For solutions without microspheres phenol measured across 24-h, 48-h and 72-h was close to the nominal concentration which is the amount of phenol which is expected to be present in the solution prepared. Measured concentrations were generally about 15% below the nominal concentrations for all test solutions without spheres. The reduction of phenol when comparing the phenol measurements between test solutions without microspheres and test solutions with microspheres was analyzed using 0ne-way ANOVA procedure, and then Fisher's LSD procedure to determine the nominal concentrations and exposure time at which the reduction of phenol was statistically significant.

Table 16.1 indicates the results for the one-way ANOVA analysis. Phenol was measured in test solutions without microspheres and with microspheres, and these measurements were arranged into groups according to the nominal concentrations and exposure times.

Based on the statistical differences found in test solutions with and without microspheres across time, it was observed that phenol started reducing after 24 h only in test solutions at the higher nominal concentrations (160 and 200 mg L^{-1}). The phenol reduction was significant only when comparing the solutions without spheres to solutions with spheres.

After 48 h phenol was reduced in the nominal concentrations at 40, 160 and 200 mg L^{-1} for the test solutions containing microspheres as it is indicated by the statistical differences found in 16.1. Phenol was only reduced significantly when comparing to the solutions without microspheres to the solutions with microspheres at the three nominal concentrations.

After 72 h phenol was reduced in the three higher nominal concentrations at 120, 160 and 200 mg L^{-1} for test solutions containing microspheres. Phenol was significantly reduced when comparing test solutions without microspheres to solutions containing microspheres, and as for 24h and 48h the statistical differences in phenol reduction observed at 72h are also summarized in 16.1.

Table 16.1: One-way ANOVA results for phenol (mean) measured concentrations (mg L^{-1}).

Time Point (hours)	Nominal (mg L^{-1})	d.f.	SS	MS	*F* ratio	*P* value
0	40	3	0.63	0.21	0.57	0.6505
	Error	8	2.95	0.37		
0	80	3	1.21	0.4	0.37	0.7774
	Error	8	8.79	1.09		
0	120	3	0.63	0.21	0.26	0.8498
	Error	8	6.45	0.80		
0	160	3	0.20	0.06	0.03	0.9919
	Error	8	17.13	2.14		
0	200	3	0.40	0.16	0.18	0.9037
	Error	8	6.96	0.87		
24	40	3	6.42	2.14	1.20	0.3693
	Error	8	14.24	1.78		
24	80	3	4.38	1.46	0.77	0.5446
	Error	8	15.27	1.90		
24	120	3	3.95	1.31	0.88	0.4915
	Error	8	11.93	1.49		
24	160	3	20.78	6.92	4.61	0.0372
	Error	8	12.01	1.50		
24	200	3	457.73	152.57	102.67	< 0.0001
	Error	8	11.88	1.48		
48	40	3	6.72	2.24	11.28	0.0030
	Error	8	1.58	0.19		
48	80	3	2.89	0.96	0.45	0.7241
	Error	8	17.15	2.14		
48	120	3	4.05	1.35	0.66	0.5994
	Error	8	16.37	2.04		
48	160	3	58.75	19.58	13.83	0.0016
	Error	8	11.31	1.41		
48	200	3	563.05	187.68	185.56	< 0.0001
	Error	8	8.09	1.01		
72	40	3	0.60	0.20	0.41	0.7530
	Error	8	3.96	0.49		
72	80	3	1.11	0.37	0.15	0.9239
	Error	8	19.23	2.40		
72	120	3	134.45	44.81	35.37	< 0.0001
	Error	8	10.13	1.26		
72	160	3	20.80	6.93	4.92	0.0318
	Error	8	11.27	1.40		
72	200	3	760.41	253.47	217.52	< 0.0001
	Error	8	9.32	1.16		

Discussion

An experimental design was developed to investigate the impact of various plastic microparticle concentration levels upon the toxicity of an organic compound (phenol) to the brine shrimp *Artemia*. The plastic material chosen was polystyrene, which is a rigid and inexpensive plastic, used as the basis for one of the most popular foamed plastic, "styrene foam" used for packing and insulating. The plastic particle size was within the particle range reported as freely suspended particles frequently captured by other filter-feeding species such bivalves (Ward & Kach, 2009; Brown et al., 2001; Oberdörster et al., 1994).

Toxicity of phenol to adult Artemia

The toxicity of phenol to brine shrimp cohorts (3-week-old *Artemia*) was determined in acute toxicity tests. Lethal effect causing 50% of mortality in the brine shrimp populations, across time, was used as the response criterion for phenol toxicity. The quantification of the 24-h, 48-h and 72-h LC50 values for phenol alone in *Artemia* is shown in Table 1.1. Difference between LC50 values for 24h-LC50 and 48h-LC50 was significant. In other words, toxicity of phenol at 24h-LC50 found to be 211.06 mg L^{-1} was less than the 48h-LC50 found to be 113.19 mg L^{-1}. However, no significant difference was determined between 48h-LC50 and 74h-LC50 values for phenol alone.

Guerra (2001) and Walker (1988) found 24-h LC50 values of phenol to *Artemia* nauplii were 17.35 and 56 mg L^{-1}, respectively. Similarly, Price et al. (1974) found 24-h LC50 and 48-h LC50 values of phenol to *Artemia* nauplii were 154 and 57 mg L^{-1},

respectively. In the present investigation, we found LC50 values that are higher than the LC50 values of phenol reported by these authors. The difference in LC50 values of phenol for *Artemia* is possibly due to the differences in sensitivities to the toxicant between young and adult stages. The LC50 values of phenol in the present investigation clearly demonstrated a significant difference in sensitivity between nauplii and adult particularly for the 24-h LC50 values reported by Guerra (2001) and Walker (1988). The 24h, 48h and 72h-LC50 values for phenol alone indicated adult *Artemia* was a less sensitive organism to the acute effects of phenol.

Adult Artemia exposed to phenol containing microplastic particles

As for the test solution of phenol alone, populations of brine shrimp *Artemia*, adult stage, were exposed to phenol solutions containing polystyrene microspheres (3-µm in diameter) to examine the impact of varying concentrations of microspheres upon the toxicity of phenol by estimating 24-h, 48-h and 72-h LC50 values from the nominal concentrations of phenol containing microsphere concentrations (dry weight of polystyrene beads in D.I. suspension) at 100, 200 and 300 mg L^{-1}.

Differences in LC50 values within test solutions were not statistically significant at 0.05 confidence level. A comparison of LC50 values across time for test solutions with microspheres and without microspheres indicated that the toxicity of phenol to the brine shrimp did not significantly change.

In contrast, when comparing LC50 values from test solutions without microspheres to solutions containing microspheres at fixed time, significant differences were found among their LC50 values. In this sense, the 24h-LC50 values from test solutions containing microspheres were greater than 24h-LC50 values from test solution without microspheres. Likewise, LC50 values from test solutions containing microsphere concentrations at 100, 200 and 300 mg L^{-1} as their 24h-LC50 values found to be 213.33, 213.72 and 216.41 mg L^{-1}, respectively, were significantly different from the 24h-LC50 found to be 211.06 mg L^{-1} for phenol alone. These results have shown a low toxicity of phenol in general at 24h upon the population of adult brine shrimp because toxicity curves for these LC50 values were also below the median lethal threshold concentration (Figs. 5.1-8.1). However, the data was further investigated in order to determine significant differences between acute toxic effects of phenol, expressed as LC50 values, in test solutions with microspheres and without microspheres.

Differences in 48h-LC50 values for test solutions containing microspheres at 100, 200 and 300 mg L^{-1} were significantly greater than the 48h-LC50 value from phenol alone. This suggested that the toxicity of phenol to the brine shrimps was lowered in test solutions containing microspheres at 48h. Moreover, significant differences were found among the solutions containing only microsphere concentrations. Thus, ranking the most toxic concentration first was found at 106.45 mg L^{-1} for 48h-LC50 in phenol at 100 mg L^{-1} > 113.19 mg L^{-1} for 48h-LC50 in phenol alone > 121.34 mg L^{-1} for 48h-LC50 in phenol with microspheres at 200 mg L^{-1} > 175.15 mg L^{-1} in phenol with microspheres at 300 mg L^{-1}. It is important to mention that 48-h LC50 values for 121.34 mg L^{-1} and 175.15 mgL^{-1}

have increased compared to the 48-h LC50 value for phenol alone, 113.19 mg L^{-1}. This suggested that the toxicity of phenol has been lowered by increased 48h-LC50 values obtained from test solutions with microspheres.

Similar to the results from 48h-LC50 values, differences in 72h-LC50 values for test solutions with the microsphere concentrations at the three nominal concentrations were significantly greater than the 72h-LC50 value from test solution without microspheres. After 72h the toxicity of phenol, in terms of LC50 values, was lowered in test solutions containing microsphere concentrations than in solution without microspheres. And ranking the most toxic concentration first was found at 90.90 mg L^{-1} for 72h-LC50 in phenol alone > 91.57 mg L^{-1} for 72h-LC50 in phenol with microspheres at 100 mg L^{-1} > 100.25 mg L^{-1} for 72h-LC50 in phenol with microspheres at 200 mg L^{-1} > 116.75 mg L^{-1} for 72h-LC50 in phenol with microspheres at 300 mg L^{-1}. Moreover, toxicity curves for the 72h-LC50 values showed a less lethal response in the brine shrimp populations being exposed to test solutions containing the microsphere concentrations.

Results from the toxicity tests showed a relationship between the increase in LC50 values and the levels of microsphere concentration tested. Data from toxicity tests showed that LC50 values found in test solutions containing microspheres were greater than LC50 values found in test solutions without microspheres. Thus, in terms of LC50 values, phenol toxicity was lowered by the addition and varying concentrations of microspheres in test solutions at phenol concentrations at which acute toxic effects were observed.

Lethal effects on the brine shrimp *Artemia* exposed to phenol solutions were observed at the higher nominal concentrations of phenol tested and after 48-h exposure. Statistical analysis was used to identify and describe significant differences among survival percentages from solutions containing microspheres at the three different concentrations. Significant differences were mainly observed in the test solutions with the two higher concentrations of microspheres for 48h and 72h exposure times.

The results of LC50 values from test solutions containing polystyrene microspheres, contrast with those concerns that the interaction of microplastics with hydrophobic pollutants cannot only act as transport of chemicals enter the environment, but also becoming more bioavailable to microorganism and enhance toxicity (e.g. acute lethal effects). The potential for plastics to transport contaminants was investigated by Gregory (1996) in a report about marine pollution, he suggested that finer-sized plastic particles dispersed through the oceanic water column could be a mean to transfer heavy metal and other contaminants (e.g. organochlorines) to filter feeding and other species of invertebrate, and ultimate to higher trophic levels.

The decrease of acute toxicity of phenol, in terms of LC50 values, to the brine shrimp *Artemia* can be attributed to the reduction of phenol and the microsphere uptake by the brine shrimp.

Phenol concentrations measured in test solutions without microspheres were used as control to determine the amount of phenol potentially lost to volatilization/degradation during toxicity testing. Consequently, additional reduction of phenol in test solutions containing polystyrene microspheres was attributed to the adsorption of phenol onto the particles as the phenol measurements from test solutions without microspheres suggest that physical volatilization or chemical degradation were not responsible for the reduction of phenol concentration. In this sense, field adsorption experiments conducted by Mato et al. (2001) determined hydrophobic sorption of PCBs and DDE on polypropylene plastic resin pellets from ambient seawater. They observed that these two pollutants were found to accumulate in plastic pellets in concentrations up to 10^5 and 10^6 times higher than surrounding seawater. They explained that polypropylene plastic resin pellets are manufactured of saturated hydrocarbon units with nonpolar surfaces that can readily adsorb hydrophobic contaminants from the environment. Similarities to this work can be pointed out in terms of plastic molecular structures. The molecular structure of polystyrene is similar to that of polypropylene, but with the methyl groups of polypropylene replaced by phenyl groups. This may suggest the same process of adsorption of phenol onto polystyrene beads due to its chemical profile.

Similarly, Teuten et al. (2007) confirmed the high affinity of plastics to sorb hydrophobic compounds by developing isotherm parameters for sorption of

phenanthrene, a priority pollutant, to three types of plastics (polyethylene, polypropylene, and PVC) and sediments. The reduction of phenol concentration from the test solution without microspheres at nominal concentration of 200 mg L^{-1} to the solution with microspheres (300 mg L^{-1} of microsphere concentration) at the same nominal concentration was about 12% after 72 h.

Data for body burden of polystyrene microspheres obtained for 24h, 48h and 72h periods, indicated the adult *Artemia* can uptake up more than 100% of body weight of microspheres, and that by 72 h animals are becoming saturated. Ward and Kach (2009) feeding polystyrene nanoparticles to two species of suspension-feeding bivalves reported the presence of fluorescent nanoparticles in the digestive gland and gut tissue 72 h after feeding. Data from the body burden of microspheres suggest a retention gut time up to 72 h since a consistent difference among dry weight values was observed over time.

The uptake of microspheres by the brine shrimp suggests that microsphere levels in the animal can interact with the phenol present in the organism's gut, making the chemical less bioavailable and in this way toxicity is lowered. Data from the measured concentrations show that the favorable action of higher microsphere concentrations in test solutions was the increase in LC50 values. The lack of particle discrimination of the brine shrimp permits the uptake of the microspheres in great quantities. Moreover, data from the body burden of microsphere suggest that microspheres start progressively move through the organism's gut as there is more pressure on it from the high microsphere concentrations in the media. Presumably, the more microspheres in the gut permits greater reduction of phenol. Finally, it is clear that there is a correlation between high

microsphere levels and toxicity response based on the increase in LC50 values computed for acute exposures.

The addition of clean polystyrene microspheres to phenol solutions seems to lower the toxicity of phenol by increasing the LC50 values among the test solutions with microspheres at different exposure times. This finding suggests that the interaction of phenol and clean polystyrene microspheres in contaminated solutions produces a "protective effect" upon the brine shrimp for acute exposures. Teuten et al. (2007) in experiments to investigate the potential for plastics to transport contaminants to organisms, also pointed out a beneficial effect of clean microplastics in reducing contaminant concentrations in benthic organisms by predicting a 13% reduction in phenanthrene tissue concentrations in a lugworm species after the addition of 81 ppm polyethylene to low % organic carbon sediment habitat. Sorption of phenol to the polystyrene microspheres is supported by other plastic congener profiles (polyethylene, polypropylene, PVCs). Results indicate that reduction of phenol in test solutions containing polystyrene microspheres was not due to any loss/volatilization over time but associated to the presence of microspheres at the various concentrations tested.

Implications and recommendations

This study presents new data to better understand the impacts of microplastics upon the toxicity of an organic pollutant, phenol, to a marine model organism, the brine shrimp *Artemia*. LC50 values of test solutions containing polystyrene microspheres indicated that the toxicity of phenol was lowered. It is suggested that microspheres are protecting the brine shrimp against the lethal effect-concentrations of phenol for acute exposures. Moreover, data from the body burn of microspheres indicated that the uptake of particles by the brine shrimp can be up to 100% of body weight after 48 h. The uptake of particles may also contribute to protect the brine shrimp by adsorption of the phenol present in the organism's gut, given the capacity of plastic to sorb hydrophobic compounds. Studies on desorption of hydrophobic compounds (Teuten et al., 2007) indicated that desorption occurred more rapidly from sediments than from plastics. The research also raises important issues relevant to microplastics in the environment. For example since the uniformity in particle shapes in the environment is unlikely to occur, it could be important to test different shapes of microplastics at relevant concentrations for the open-ocean environment. Adsorption/desorption in polystyrene beads should be also described so models to estimate the concentration of phenol in microspheres can be developed. Finally, body burden of phenol for the brine shrimp should be also determined so concentrations of phenol in other phases can be developed.

Conclusions

1. The 24h-LC50 values and their toxicity curves for test solutions with polystyrene microspheres and without microspheres were below the lethal threshold concentration and indicated that no mortality at 50% in the adult *Artemia* population could be observed at 24h.
2. Differences in LC50 values within test solutions with microspheres and without microspheres did not show a significant difference across time.
3. Differences in LC50 values among test solutions with microspheres and without microspheres at the exposure times employed were significant, which suggested that toxicity of phenol to the brine shrimp was lowered due to the addition of polystyrene microspheres.
4. Body burden of microspheres was related to exposure concentrations across time.
5. The sorption of phenol to polystyrene microspheres is supported by other plastic congener profiles protecting the protecting the brine shrimp against toxic levels of phenol. Moreover, the binding between the phenol and the polystyrene microspheres may be attributed to their mutual hydrophobic interaction as well as the pi-stacking between the aromatic moieties of the two species.

APPENDICES

Appendix A

Survival Tables for 24h, 48 and 72h Toxicity Tests

Replicate	Toxicant	Trial	Nominal Concentration (mg L^{-1})	n	24 h Observations		48 h Observations		72 h Observations	
					Alive	Dead	Alive	Dead	Alive	Dead
r1	Phenol	1	40	10	10	0	9	1	9	1
	Phenol	1	80	10	10	0	9	1	9	1
	Phenol	1	120	10	7	3	5	5	3	7
	Phenol	1	160	10	7	3	4	6	2	8
	Phenol	1	200	10	7	3	1	9	0	10
	Control	1	0	10	10	0	10	0	10	0
r2	Phenol	2	40	10	10	0	10	0	10	0
	Phenol	2	80	10	9	1	9	1	9	1
	Phenol	2	120	10	8	2	5	5	3	7
	Phenol	2	160	10	7	3	4	6	2	8
	Phenol	2	200	10	7	3	1	9	0	10
	Control	2	0	10	10	0	10	0	9	1
r3	Phenol	3	40	10	10	0	10	0	10	0
	Phenol	3	80	10	10	0	8	2	8	2
	Phenol	3	120	10	8	2	6	4	2	8
	Phenol	3	160	10	6	4	3	7	1	9
	Phenol	3	200	10	6	4	0	10	0	10
	Control	3	0		10	0	10	0	10	0

Table A-1: Survival data for 24h, 48h and 72h phenol alone toxicity tests.

Replicate	Toxicant	Trial	Nominal Concentrations (mg L^{-1})	n	24 h Observations		48 h Observations		72 h Observations	
					Alive	Dead	Alive	Dead	Alive	Dead
r1	Phenol + microsphere	1	40 + 100	10	10	0	10	0	10	0
	Phenol + microsphere	1	80 + 100	10	10	0	8	2	8	2
	Phenol + microsphere	1	120 + 100	10	8	2	4	6	3	7
	Phenol + microsphere	1	160 + 100	10	6	4	3	7	2	8
	Phenol + microsphere	1	200 + 100	10	7	3	2	8	0	10
	Control	1	0	10	10	0	10	0	10	0
r2	Phenol + microsphere	2	40 + 100	10	10	0	10	0	10	0
	Phenol + microsphere	2	80 + 100	10	10	0	9	1	9	1
	Phenol + microsphere	2	120 + 100	10	8	2	4	6	3	7
	Phenol + microsphere	2	160 + 100	10	7	3	3	7	2	8
	Phenol + microsphere	2	200 + 100	10	7	3	2	8	0	10
	Control	2	0	10	10	0	10	0	10	0
r3	Phenol + microsphere	3	40 + 100	10	10	0	9	1	9	1
	Phenol + microsphere	3	80 + 100	10	9	1	9	1	9	1
	Phenol + microsphere	3	120 + 100	10	7	3	4	6	3	7
	Phenol + microsphere	3	160 + 100	10	7	3	3	7	2	8
	Phenol + microsphere	3	200 + 100	10	7	3	1	9	0	10
	Control	3	0		10	0	10	0	10	0

Table A-2: Survival data for 24h, 48h and 72h phenol and microsphere concentration at 100 mg L^{-1} toxicity tests.

Replicate	Toxicant	Trial	Nominal Concentrations (mg L^{-1})	n	24 h Observations		48 h Observations		72 h Observations	
					Alive	Dead	Alive	Dead	Alive	Dead
r1	Phenol + microsphere	1	40 + 200	10	10	0	10	0	10	0
	Phenol + microsphere	1	80 + 200	10	10	0	9	1	9	1
	Phenol + microsphere	1	120 + 200	10	9	1	5	5	3	7
	Phenol + microsphere	1	160 + 200	10	8	2	4	6	3	7
	Phenol + microsphere	1	200 + 200	10	7	3	2	8	1	9
	Control	1	0	10	10	0	10	0	10	0
r2	Phenol + microsphere	2	40 + 200	10	10	0	10	0	10	0
	Phenol + microsphere	2	80 + 200	10	10	0	9	1	9	1
	Phenol + microsphere	2	120 + 200	10	8	2	5	5	3	7
	Phenol + microsphere	2	160 + 200	10	8	2	4	6	3	7
	Phenol + microsphere	2	200 + 200	10	7	3	2	8	1	9
	Control	2	0	10	10	0	10	0	10	0
r3	Phenol + microsphere	3	40 + 200	10	10	0	10	0	10	0
	Phenol + microsphere	3	80 + 200	10	10	0	9	1	9	1
	Phenol + microsphere	3	120 + 200	10	8	2	6	4	3	7
	Phenol + microsphere	3	160 + 200	10	7	3	4	6	3	7
	Phenol + microsphere	3	200 + 200	10	8	2	3	7	1	9
	Control	3	0		10	0	10	0	10	0

Table A-3: Survival data for 24h, 48h and 72h phenol and microsphere concentration at 200 mg L^{-1} toxicity tests.

Replicate	Toxicant	Trial	Nominal Concentrations (mg L^{-1})	n	24 h Observations		48 h Observations		72 h Observations	
					Alive	Dead	Alive	Dead	Alive	Dead
r1	Phenol + microsphere	1	40 + 300	10	10	0	10	0	10	0
	Phenol + microsphere	1	80 + 300	10	10	0	9	1	9	1
	Phenol + microsphere	1	120 + 300	10	8	2	7	3	5	5
	Phenol + microsphere	1	160 + 300	10	8	2	6	4	5	5
	Phenol + microsphere	1	200 + 300	10	7	3	6	4	2	8
	Control	1	0	10	10	0	10	0	10	0
r2	Phenol + microsphere	2	40 + 300	10	10	0	10	0	10	0
	Phenol + microsphere	2	80 + 300	10	10	0	9	1	9	1
	Phenol + microsphere	2	120 + 300	10	8	2	7	3	5	5
	Phenol + microsphere	2	160 + 300	10	8	2	6	4	5	5
	Phenol + microsphere	2	200 + 300	10	8	2	6	4	2	8
	Control	2	0	10	10	0	10	0	10	0
r3	Phenol + microsphere	3	40 + 300	10	10	0	10	0	10	0
	Phenol + microsphere	3	80 + 300	10	10	0	9	1	9	1
	Phenol + microsphere	3	120 + 300	10	9	1	7	3	5	5
	Phenol + microsphere	3	160 + 300	10	8	2	6	4	5	5
	Phenol + microsphere	3	200 + 300	10	7	3	6	4	2	8
	Control	3	0		10	0	10	0	10	0

Table A-4: Survival data for 24h, 48h and 72h phenol and microsphere concentration at 300 mg L^{-1} toxicity tests.

Appendix B

Phenol Measured Concentrations in Test Solutions

Exposure Time (hours)	Phenol Concentrations		
	Nominal (mg L^{-1})	Microsphere Concentration (mg L^{-1})	Mean Measured with S.E.
0 h	40	---	39.17 (0.24)
	40	100	28.85 (0.23)
	40	200	38.80 (0.51)
	40	300	38.52 (0.34)
	80	---	80.53 (0.45)
	80	100	80.02 (0.51)
	80	200	79.80 (0.66)
	80	300	79.71 (0.73)
	120	---	117.0 (0.54)
	120	100	116.70 (0.58)
	120	200	116.50 (0.54)
	120	300	116.40 (0.38)
	160	---	147.37 (0.27)
	160	100	147.11 (1.27)
	160	200	147.10 (0.63)
	160	300	147.02 (0.87)
	200	---	187.50 (0.60)
	200	100	187.14 (0.45)
	200	200	187.02 (0.39)
	200	300	187.0 (0.65)

Table B-1: Phenol measured in test solutions at 0 h.

Exposure Time (hours)	Phenol Concentrations		
	Nominal (mg L^{-1})	Microsphere Concentration (mg L^{-1})	Mean Measured with S.E.
24 h	40	---	37.07 (0.22)
	40	100	37.01 (0.70)
	40	200	35.41 (1.24)
	40	300	35.81 (0.53)
	80	---	73.01 (0.22)
	80	100	72.83 (0.25)
	80	200	72.03 (1.01)
	80	300	71.51 (1.81)
	120	---	115.17 (0.44)
	120	100	115.07 (0.57)
	120	200	114.20 (0.95)
	120	300	113.81 (0.73)
	160	---	146.45 (0.27)
	160	100	144.04 (0.87)
	160	200	143.53 (0.48)
	160	300	143.02 (0.96)
	200	---	183.28 (1.05)
	200	100	179.02 (0.34)
	200	200	169.93 (0.54)
	200	300	168.50 (0.66)

Table B-2: Phenol measured in test solutions at 24 h.

Exposure Time (hours)	Phenol Concentrations		
	Nominal (mg L^{-1})	**Microsphere Concentration (mg L^{-1})**	**Mean Measured with S.E.**
48 h	40	---	34.07 (0.12)
	40	100	33.31 (0.31)
	40	200	32.75 (0.12)
	40	300	32.03 (0.36)
	80	---	67.15 (0.22)
	80	100	66.20 (0.67)
	80	200	66.01 (1.42)
	80	300	65.91 (0.57)
	120	---	113.02 (0.44)
	120	100	112.0 (1.06)
	120	200	111.71 (1.10)
	120	300	111.51 (0.40)
	160	---	145.02 (0.35)
	160	100	143.50 (0.86)
	160	200	142.01 (0.94)
	160	300	139.03 (0.34)
	200	---	182.27 (0.29)
	200	100	175.0 (0.06)
	200	200	167.03 (0.74)
	200	300	165.0 (0.83)

Table B-3: Phenol measured in test solutions at 48 h.

Exposure Time (hours)	Phenol Concentrations		
	Nominal (mg L^{-1})	**Microsphere Concentration (mg L^{-1})**	**Mean Measured with S.E.**
72 h	40	---	32.05 (0.21)
	40	100	31.50 (0.28)
	40	200	31.61 (0.58)
	40	300	31.50 (0.42)
	80	---	63.59 (0.42)
	80	100	62.91 (0.47)
	80	200	63.01 (1.57)
	80	300	62.81 (0.55)
	120	---	112.10 (0.36)
	120	100	110.04 (0.57)
	120	200	107.60 (0.55)
	120	300	103.12 (0.96)
	160	---	143.08 (0.44)
	160	100	141.07 (0.99)
	160	200	140.03 (0.57)
	160	300	139.71 (0.59)
	200	---	179.57 (0.44)
	200	100	171.06 (0.59)
	200	200	165.92 (0.84)
	200	300	157.91 (0.53)

Table B-4: Phenol measured in test solutions at 72 h.

Appendix C

Individual Brine Shrimp Dry Weight

Individual dry weight (mg) at 24 h							
Weights	Brine Individuals Exposed to Microsphere Concentrations (S) at 0, 100, 200, 300 mg L^{-1}						
	S-0	S-100	*Differences between S-0 and S-100*	S-200	*Differences between S-100 and S-200*	S-300	*Differences between S-200 and S-300*
sample 1	0.43	0.49	0.06	0.54	0.11	0.62	0.19
sample 2	0.45	0.50	0.05	0.55	0.10	0.62	0.17
sample 3	0.44	0.49	0.05	0.56	0.12	0.63	0.19
sample 4	0.43	0.50	0.07	0.54	0.11	0.62	0.19
sample 5	0.43	0.50	0.07	0.54	0.11	0.62	0.19
Std. Dev.	0.0089	0.55		0.0089		0.0045	
Variance	$8x10^{-5}$	$3x10^{-5}$		$8x10^{-5}$		$2x10^{-5}$	

Table C-1: Dry weight of brine shrimps exposed to microsphere concentrations only.

Individual dry weight (mg) at 48 h							
Weights	Brine Individuals Exposed to Microsphere Concentrations (S) at 0, 100, 200, 300 mg L^{-1}						
	S-0	S-100	*Differences between S-0 and S-100*	S-200	*Differences between S-100 and S-200*	S-300	*Differences between S-200 and S-300*
sample 1	0.38	0.54	0.16	0.70	0.32	0.91	0.53
sample 2	0.39	0.54	0.15	0.69	0.30	0.90	0.51
sample 3	0.38	0.55	0.17	0.71	0.33	0.92	0.54
sample 4	0.39	0.54	0.15	0.71	0.32	0.90	0.51
sample 5	0.39	0.54	0.15	0.71	0.32	0.90	0.51
Std. Dev.	0.0055	0..0045		0.0089		0.0089	
Variance	$3x10^{-5}$	$2x10^{-5}$		$8x10^{-5}$		$8x10^{-5}$	

Table C-2: Dry weight of brine shrimps exposed to microsphere concentrations only after 48 h.

Individual dry weight (mg) at 72 h							
Weights	Brine Individuals Exposed to Microsphere Concentrations (S) at 0, 100, 200, 300 mg L^{-1}						
	S-0	S-100	*Differences between S-0 and S-100*	S-200	*Differences between S-100 and S-200*	S-300	*Differences between S-200 and S-300*
sample 1	0.32	0.83	0.51	0.87	0.55	1.05	0.73
sample 2	0.31	0.82	0.51	0.88	0.57	1.00	0.69
sample 3	0.32	0.84	0.52	0.86	0.54	1.10	0.78
sample 4	0.31	0.83	0.52	0.87	0.56	1.05	0.74
sample 5	0.31	0.83	0.52	0.87	0.56	1.05	0.74
Std. Dev.	0.0055	0.01		0.01		0.04	
Variance	$3x10^{-5}$	$5x10^{-5}$		$5x10^{-5}$		$1.25x10^{-3}$	

Table C-3: Dry weight of brine shrimps exposed to microsphere concentrations only after 72 h.

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