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Toxicity of Synthetic Musks to Early Life Stages of the Freshwater Mussel *Lampsilis cardium*

M.P. Gooding[1,*], **T.J. Newton**[2], **M.R. Bartsch**[2], and **K.C. Hornbuckle**[1,3,*]

[1]Center for Global and Regional Environmental Research, University of Iowa, 424 Iowa Advanced Technology Laboratories, Iowa City, IA 52242, USA

[2]U.S. Geological Survey, Upper Midwest Environmental Sciences Center, 2630 Fanta Reed Road, La Crosse, WI 54603, USA

[3]Department of Civil and Environmental Engineering, University of Iowa, Iowa City, IA 52242, USA

Abstract

Polycyclic musk fragrances are common additives to many consumer products. As a result of their widespread use and slow degradation rates, they are widely found in aquatic environments. This study reports on the lethal and sublethal toxicity of the polycyclic musks AHTN (Tonalide®) and HHCB (Galaxolide®) to glochidial (larval) and juvenile life stages of the freshwater mussel *Lampsilis cardium* (Rafinesque, 1820). In glochidia, 24-h median lethal concentrations (LC50s) ranged from 454 to 850 μg AHTN/L and from 1000 to >1750 μg HHCB/L (water solubility). Results for 48-h tests were similar to the 24-h tests. In 96-h tests with juveniles, we did not observe a dose-response relation between mortality and either musk. However, growth rate was reduced by musk exposure. The median effective concentrations (EC50s, based on growth) were highly variable and ranged from 108 to 1034 μg AHTN/L and 153 to 831 μg HHCB/L. While all adverse effects occurred at concentrations that are much greater than those reported in natural waters (low μg/L to ng/L), these results indicate the potential for adverse effects on these long-lived organisms from exposure to synthetic musk fragrances.

Introduction

Synthetic musks are fragrance additives used in soaps, perfumes, cosmetics, air fresheners, laundry detergents, and shampoos. These products are ubiquitous in the aquatic environment due to disposal of musk-containing products via water systems and incomplete removal by sewage treatment (Bester 2005; Eschke 2004). The estimated consumption of fragrance chemicals in the United States, including synthetic musk fragrances, benzenoids, terpenes, and other fragrance compounds, has more than doubled since 1990 (Somogyi and Kishi 2001). The musk fragrances with the highest volume of use are the polycyclic musks, of which two compounds, AHTN (Tonalide®, 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene) and HHCB (Galaxolide®, 1,3,4,6,7,8-hexahydro-4,6,6,7,8-hexamethylcyclopenta-γ-2-benzopyran), represented 95% of total polycyclic musk production in 1996 (Rimkus 1999). The physiochemical properties and octanol-water partition coefficients (log K_{ow}) of AHTN (5.7) and HHCB (5.9) suggest that these compounds will bioaccumulate (Balk and Ford 1999). Indeed, AHTN and HHCB have been detected in human breast milk

Corresponding author: Keri C. Hornbuckle, Department of Civil and Environmental Engineering, University of Iowa, Iowa City, IA 52242, USA, phone: 319-384-0789, FAX: 319-335-5660, keri-hornbuckle@uiowa.edu.
**Present address*: Winona State University, Department of Biology, Winona, MN 55987, USA

and adipose tissue (Liebl et al. 2000; Rimkus and Wolf 1996; Zehringer and Herrmann 2001), fish, mussels, shrimp, and otters (Leonards and deBoer 2004).

In aquatic ecosystems, concentrations of musks decline with increasing distance from wastewater treatment plants. For example, concentrations of AHTN and HHCB in Western European surface waters were generally <1 μg/L (as cited in Balk et al. 2001) and were 0.047 (HHCB) and 0.001 (AHTN) μg/L in Lake Michigan (Peck and Hornbuckle 2004). In a study of 12 sewage treatment plants in the U.S., including plants with different treatment processes, Simonich et al. (2002) reported mean AHTN and HHCB concentrations in effluents at 1.2 and 1.6 μg/L, respectively. Exceptionally high values of 6.8 (AHTN) and 13.3 (HHCB) μg/L were reported in a small brook in Germany that consisted primarily of sewage effluent (Heberer et al. 1999). Because they are incompletely removed during the wastewater treatment process, AHTN and HHCB are presumably consistent components of contemporary municipal waste discharge. Lastly, although ~98% of AHTN and HHCB are found in the dissolved phase of dilute waters (Peck and Hornbuckle 2004), musks have also been detected in suspended particulate matter and sediments (Fooken 2004).

Many synthetic musks are not regarded as acutely toxic to aquatic organisms given that LC50s (median lethal concentrations resulting in 50% mortality) are generally several orders of magnitude higher than environmental concentrations (Balk et al. 2001). However, sublethal effects can occur at concentrations that approach environmentally relevant concentrations. For example, Breitholtz et al. (2003) observed impairment of larval development in the copepod *Nitocra spinipes* at concentrations as low as 20 μg HHCB/L. Inhibition of larval development in the copepod *Acartia tonsa* was observed at 26 μg AHTN/L and 59 μg HHCB/L (Wollenberger et al. 2003). Recently, Luckenbach et al. (2004) demonstrated that four polycyclic musks, including AHTN and HHCB, compromised multixenobiotic defense systems (mxr transporters) in the marine mussel *Mytilus californianus* in the μM range (200-2000 μg/L). A long-term loss of efflux transporter activity can result in continued accumulation of normally excluded toxicants even after direct exposure to the musk has ended, and thus, may increase an organisms susceptibility to other toxicants (Luckenbach and Epel 2005).

Freshwater mussels (Family Unionidae) are among those species that may be the most vulnerable to chronic exposure to polycyclic musks. Mussels are found in areas that are affected by wastewater outflows, they live in sediments, they are filter feeders, and their life cycle includes early life stages that may be vulnerable to the effects of xenobiotics. Glochidia (larvae) of most freshwater mussel species are dependent upon a fish host to continue their life cycle; glochidia that do not attach to a fish are not viable. Freshwater mussels are the most imperiled group of organisms in North America, with 67% classified as vulnerable, extinct, or threatened (Bogan 1993; Williams et al. 1993). Freshwater mussels may also be good indicators for ecosystem changes associated with environmental contaminants because they are long-lived, low on the aquatic food chain, exposed to sediment-associated contaminants, and bioaccumulate pollutants (Naimo 1995). They are also relatively sensitive to a variety of organic and inorganic toxicants, compared to traditionally tested organisms (Naiman et al. 1995; Naimo 1995).

Recently, a standard guide for conducting laboratory tests with the early life history stages of freshwater mussels has become available (ASTM 2006). Because of the small size of the larvae (~300 μm), most toxicity studies use survival as the test endpoint (Newton et al. 2003; ASTM 2006), although most researchers recognize the need to develop sublethal biomarkers for these animals (Newton and Cope in press). In this study, the effect of synthetic musk exposure on juvenile growth was evaluated to assess the effectiveness of using this sublethal endpoint as an indicator of toxicity. A series of experiments were also conducted to evaluate the lethal

effects of HHCB and AHTN to glochidial (larval) and juvenile life stages in a common freshwater mussel species.

Methods

Chemicals

The polycyclic musks AHTN (purity 98%) and HHCB were purchased from Prochem (Teddington, UK). The HHCB technical product contains 26% diethylphtalate but this compound was not quantified in our samples. All solvents used were Fisher Scientific Pesticide Grade (Fair Lawn, NJ, USA). All glassware were combusted at 450°C for at least 4 h prior to use.

Test organisms

Lampsilis cardium (Rafinesque, 1820) is common throughout the eastern half of North America and may be a surrogate for the federally endangered *Lampsilis higginsii*, because both occupy similar habitats and belong to the same genus (Newton et al. 2003). Gravid *L. cardium* were obtained from the Boone River in Hamilton County, IA, transported to the laboratory, and held at 12 to 14°C in a flow-through tank containing sand and dechlorinated tap water. Mussels were fed a commercial shellfish diet (Instant Algae Shellfish Diet, Reed Mariculture, San Jose, CA, USA) three times weekly at a ration of 1.2 mL/mussel/d. Mature glochidia (−300 μm) were removed from the gills of female mussels following Waller and Holland-Bartels (1988). A subsample of glochidia was tested for viability following Jacobson et al. (1997). All tests were conducted using a composite of glochidia from the same three females to enhance genetic variation and to remove potential variability associated with using different batches of glochidia.

Toxicity tests with glochidia

Replicate 24- and 48-h static tests with glochidia were conducted with HHCB and AHTN. For each test, we exposed glochidia to one of three nominal musk concentrations (400, 800, and 1600 μg HHCB/L or 300, 600, and 1200 μg AHTN/L), a well water control, and an acetone solvent control. Each experiment was run twice with four replicates per concentration. The highest concentration in each test was near the water solubility limit for both chemicals (1250 μg AHTN/L, 1750 μg HHCB/L). High concentrations of musks were used in these experiments because there was no previous data on unionid sensitivity to synthetic fragrances and the purpose of this study was to examine their relative sensitivity. Stock solutions were prepared in acetone just before use. The 24- and 48-h tests were initiated at the same time with the same stock solutions. Test solutions were prepared by adding the appropriate stock solution to 1 L of aerated well water and stirring the solution for 30 min. Acetone concentrations did not exceed 0.005% of the total volume in any treatment. Each experimental unit (EU, 250-mL beaker) received 200 mL of solution and 25 glochidia that were suspended in a 4.4-cm ID glass cylinder with a 153 μm mesh bottom. Each EU was covered with a petri dish to reduce volatilization.

All tests were conducted in an incubator at 20°C under 16 h light: 8 h dark. After 24 or 48 h, the viability of glochidia in each EU was assessed by counting the number that did not close when a saturated NaCl solution was added (Jacobson et al. 1997). These glochidia are not considered viable because they presumably are unable to attach to a fish host. The dissolved oxygen, pH, and temperature was measured daily in each EU and alkalinity, hardness, and conductivity were measured in at least two EUs per concentration at the beginning and end of each test.

Toxicity tests with juveniles

Replicate 96-h static renewal toxicity tests were conducted with juvenile *L. cardium*. To obtain juveniles, glochidia from three females were used to infect −12 largemouth bass (*Micropterus salmoides*, 6 to 15 cm long) as described in Waller and Holland-Bartels (1988). Encysted fish were held for 15 to 20 d in 38-L flow-through aquaria containing dechlorinated well water at 22°C. Encysted fish were fed fathead minnows, *Pimephales promelas, ad libitum* until juveniles began to excyst. To determine post-excystment age, aquaria bottoms were siphoned daily through a 153-μm sieve and the contents examined under a microscope. Juveniles from a given day were transferred to 7.5-cm glass cylinders with a 153 μm mesh bottom and suspended in a 38-L flow-through aquaria at 22°C until testing. For each test, 5-d-old juveniles were used. An initial subsample of at least 30 juveniles was preserved in 70% ethanol for later growth analyses.

Fifteen juveniles were randomly allocated into 7.5-cm glass cylinders with a 153 μm mesh bottom and then placed into an EU (250 mL beaker) containing ~200 mL of musk solution. Solutions were prepared as stated previously. In all tests, juveniles were exposed to 1 of 12 nominal musk concentrations (50, 100, 200, 400, 800, and 1600 μg HHCB/L or 38, 75, 150, 300, 600, and 1200 μg AHTN/L), a well water control, and an acetone solvent control; each concentration was tested in quadruplicate. Musk solutions were renewed daily. All tests were conducted at 20°C under 16 h light: 8 h dark. The dissolved oxygen, pH, and temperature was measured daily in each EU and alkalinity, hardness, and conductivity were measured in triplicate at the beginning of each test and in at least two randomly selected EUs per concentration at the end of each test.

Juvenile mussels were not fed during exposures. At the end of each test, juveniles were classified as alive or dead (Newton et al. 2003). Alive juveniles are those whose foot can be seen moving either inside or outside the shell or ciliary activity was observed. Shell height was measured in juveniles that were alive at the end of each test using an optical imaging system (Nikon Eclipse E600 Image Pro® Express, Media Cybernetics, Inc., Silver Spring, MD, USA). Growth rate (μm/d) was calculated as the difference between the mean juvenile height from a given EU and the mean juvenile height of the initial subsample, divided by the days of exposure.

Musk extraction and analysis

We removed a 10-mL water sample from each stock solution at the beginning of each test for musk analysis; these represent measured initial concentrations. Final concentrations were analyzed in three EUs per concentration in one of the replicate tests for each life stage, test duration, and musk. Additionally, during one of the juvenile tests, we removed a 10-mL sample at 0, 3, 6, 12, and 24 h from three EUs in the highest test concentration for each musk to determine loss rates over 24 h. All samples were held at 4°C and extracted within 4 weeks.

Each sample was extracted with 3-mL (1.5 mL × 2) cyclohexane with 1.08 ng/μL pentachloronitrobenzene (Chem Service,West Chester, PA, USA) as the internal standard. All samples were analyzed by a Finnigan MAT GCQ gas chromatograph-mass spectrometer system capable of MS/MS analysis equipped with a standard split/splitless injector and a CTC A200S liquid autosampler (San Jose, CA, USA). The instrument was operated in full scan mode over a small m/z (236-238). A 30-m 5% phenyl methyl siloxane capillary column (HP-5; 30 m × 0.52 mm, 0.25 μm film thickness; J & W Scientific, Folsom, CA, USA) and a constant 40 cm/min column flow rate were used. The initial GC oven temperature (90°C) was held for 2 min. This was followed by a 10°C/min ramp to 150°C, then 1.50°C/min to 170°C, and finally 30°C/min to 300°C. The final temperature (300°C) was held for 7 min. The mass spectrometer transfer line was 250°C and the source 230°C. The internal standard method was used for

quantification of all compounds. The HHCB and AHTN quantification ion was 243 (Peck and Hornbuckle 2004). The internal standard quantification ion was 237.

Prior studies have shown that musk concentrations can drop precipitously during laboratory exposures (Breitholtz et al. 2003; Schreurs et al. 2004), presumably due to volatilization, adsorption onto glass, or accumulation by biota. For example, Breitholtz et al. (2003) found that only 2 to 19% of AHTN and HHCB remained after 18 d, even though solutions were renewed every second day. This makes it difficult to quantify the actual musk concentrations that the mussels were exposed to. Thus, we report the measured musk concentration in two ways. First, we report effects based solely on the initial measured concentration of each musk (measured when the stock solutions were prepared). Second, we report effects based on the average of the initial and final measured concentrations. For those tests where we did not measure final concentrations, we multiplied the initial concentration by the percent of each musk remaining at 24 h, based on tests in which final concentrations were measured. We used the percent remaining at 24 h because this was the renewal interval in the juvenile tests.

Statistics

LC50s and associated 95% confidence limits were calculated based on both initial and average musk concentrations using probit analysis (ASTM 1989). Growth rate was analyzed using generalized linear models (McCullagh and Nelder 1989) using methods in Newton et al. (2003). The musk concentration equivalent to a 50% reduction in growth rate relative to controls (EC50), was calculated by $[\text{musk}]_{100p} = (\ln p/b_1)$ where p is the predicted growth for some fraction of the growth rate of the controls (i.e., 0.50) and b_1 is the slope of the regression line. Wald-type confidence intervals were calculated based on a delta-method approximation to the variance of EC50.

Results

Chemical analysis of musk fragrances

In tests with glochidia, the average AHTN and HHCB concentrations were 52-64% and 53-64%, respectively, of the initial concentrations (Table 1). In tests with juveniles, average concentrations were 64-65% for AHTN and 68-69% for HHCB of initial concentrations (Table 2). During our time series of musk loss in one of the juvenile tests, most of the loss of HHCB and AHTN occurred within the first 3 h (Table 3). After 3 h, only 46 to 63% of the initial concentration remained; after 24 h, 31 to 39% of the initial concentration remained. These results were similar to the 24-h glochidial tests where 26% of AHTN and 55% of HHCB remained at the end of the test (data not shown). In 48-h tests with glochidia, 8 to 19% of initial test concentrations remained at the end of the test (data not shown).

Toxicity of musks to glochidia

Water quality measures, presented as a range of means (temperature, 19.3-20.6°C; dissolved oxygen, 9.0-9.5 mg/L; pH, 8.0-8.2; alkalinity, 89-95 mg $CaCO_3$/L; hardness, 106-127 mg $CaCO_3$/L; and conductivity, 285-365 μS/cm), did not vary significantly between replicate tests. We observed a dose response relation between glochidial viability and concentrations of both AHTN and HHCB. In tests with AHTN, the range of 24-h LC50s in replicate tests was 454 to 850 μg/L and the range of 48-h LC50s was 281 to 1181 μg/L (Fig. 1). Results for tests with HHCB were considerably more variable than with AHTN. With HHCB, 24-h LC50s were 1000 μg/L to >1750 μg/L (water solubility) and 48-h LC50s were 999 μg/L to >1750 μg/L (Fig. 2). For both musks, there were no substantial differences in toxicities between the 24- and 48-h exposures, as indicated by overlapping confidence limits, probably because of the large loss of musk from solution within 24 h.

Toxicity of musks to juveniles

Water quality measures, presented as a range of means (temperature, 20.3-20.6°C; dissolved oxygen, 7.4-8.4 mg/L; pH, 8.4; alkalinity, 128-133 mg $CaCO_3$/L; hardness, 179 mg $CaCO_3$/L; and conductivity, 378-401 μS/cm), did not vary significantly between replicate tests. Musk fragrances did not exhibit a dose-response relation with mortality of juveniles at the concentrations tested (Fig. 3). After 96 h, mean mortality of juveniles across all treatments ranged from 0 to 24%. However, we observed a dose-response relation between the growth rate of juveniles and both musk fragrances. In tests with AHTN, EC50s varied over an order of magnitude and ranged from 108 to 1034 μg/L (Fig. 4). In tests with HHCB, EC50s varied from 153 to 831 μg/L (Fig. 4). In tests with both musks, EC50s were 73 (HHCB) to −84% (AHTN) lower during the second replicate test (filled circles in Fig. 4).

Discussion

Evaluation of the toxicity of synthetic musks to early life stages in freshwater mussels was complicated by variable concentrations of the musks in the test solutions. Because of the high lipophilicity and volatility of the musk compounds, AHTN and HHCB may have adsorbed to the glass in the EU or to the mesh in the chamber containing organisms, been taken up by the organisms, or been volatilized. The high initial loss rate (within 3 h) followed by a gradual decrease in concentration is consistent with rapid surface adsorption and slower volatilization, although the mechanism for loss was not determined. Regardless of the mechanism, loss rates observed in this study were consistent with previous tests with these compounds. For example, in a 96-h study with zebrafish (*Danio rerio*), 10 to 35% of the initial concentrations of AHTN and HHCB were lost following addition of the solution to test beakers without fish (Schreurs et al. 2004). Similar patterns of musk loss were observed in tests with the copepod *N. spinipes* (Breitholtz et al. 2003). Because of the potential for musk concentrations to vary over time, we reported all effects based on both initial and average concentrations to encompass the range of expected musk concentrations that mussels may have been exposed to during testing.

AHTN was considerably more toxic to glochidia than HHCB. Results from this study were similar to studies with marine copepods. For example, Breitholtz et al. (2003) reported a 96-h LC50 of 610 μg AHTN/L for adult copepods and Wollenberger et al. (2003) who reported a 48-h LC50 of 710 μg AHTN/L in the larval copepod *A. tonsa*. Data for HHCB in this study were similar to Breitholtz et al. (2003) who reported a 96-h LC50 of 1900 μg HHCB/L for *N. spinipes*, but were substantially greater than the 48-h LC50 of 470 μg HHCB/L in larval *A. tonsa* (Wollenberger et al. 2003). Data on the effects of synthetic musks to other aquatic biota are limited, but effects (EC50 or LC50) were observed in *Daphnia magna*, *Oncoryhnchus mykiss*, and *Danio rerio* in the range of 244 to 314 μg AHTN/L and 282 to 452 μg HHCB/L during 21-d exposures (Dietrich and Hitzfeld 2004). Glochidia and juvenile mussels were not as sensitive to HHCB as the copepod *N. spinipes*, where larval development rate was impaired at concentrations as low as 20 μg/L (Breitholtz et al. 2003).

Juvenile mussels exhibited no significant mortality to either synthetic musk over the 96-h duration. However, sublethal effects were observed. Growth rate has been used with success in tests with juvenile mussels and ammonia (Newton et al. 2003). In this study, growth rates were considerably lower in the second toxicity test for both musks. It is unclear why juveniles grew slower in the second test. It is possible that because the second replicate test was 6 weeks after the first test, that the physiological condition of the adults (and subsequently the juveniles) may have influenced test results. However, Newton et al. (2003) reported similar growth rates for juvenile *L. cardium* in replicate 96-h tests with ammonia, even though the tests were conducted with females held in the laboratory for 2 and 11 months prior to testing. Fish that were encysted with glochidia were obtained from the same stock, however, individual differences in the fish hosts could have affected viability of the mussels

There are few data on the life history requirements for juvenile mussels and particularly how to keep test organisms alive under laboratory conditions. It is possible that the mussels are responding to a more generalized stress condition and addition of a toxicant makes them more susceptible. However, the excellent survival of juveniles in these tests and lack of a dose-response suggests that juvenile mussels are not overly sensitive to laboratory conditions and further supports their use as test organisms. The variability observed in both the glochidial and juvenile studies cannot be explained by laboratory conditions or experimental design. All replicate tests were conducted under the same conditions. Water quality parameters (i.e., dissolved oxygen, temperature, pH, alkalinity, hardness, conductivity) were monitored in all tests and did not vary significantly between tests. In previous studies with mussels it is not uncommon for the LC50s for the same species to vary by a factor of five or more (USEPA 1999). In tests using the juvenile mussel *Utterbackia imbecilis* 96-h LC50 values ranged from 2.7 to 9.7 mg TAN at pH 8/L representing a 7-fold variation in the LC50 values (Augspurger et al. 2003).

Physiological responses observed in this study occur at concentrations several orders of magnitude higher than present environmental levels of polycyclic musks. Sewage effluents in Europe have the highest reported concentration of polycyclic musks measured in the low μg/L range (Eschke 2004). Concentrations of musks in surface waters is related to the distance from wastewater treatment plants (Balk and Ford 1999); thus, mussels that reside in proximity to sewage outfalls may be exposed to higher concentrations of musk fragrances.

Recent data suggest that these compounds are ubiquitous, albeit at low concentrations, in natural waters at locations that are remote from effluent inputs. Peck and Hornbuckle (2004) report average concentrations of HHCB and AHTN in Lake Michigan at 4.7 and 1.0 ng/L, respectively, and concentrations in Lake Mead average 0.4 ng HHCB/L and 0.2 ng AHTN/L (Osemwengie and Gerstenberger 2004). In a recent survey of wastewater contaminants in streams upstream and downstream of selected towns in Iowa, concentrations of synthetic musks varied with flow conditions and ranged from 500 to 1200 ng AHTN/L and 56 to 260 ng HHCB/L (Kolpin et al. 2004). Detection frequencies at low-flow conditions for AHTN and HHCB were 36.7% and 20%, respectively. Due to their long life span (30-100 years) and filter and deposit feeding life styles, mussels may be continually exposed to low levels of synthetic musks. It is unknown if exposure over a several year period of early development in a long-lived organism may adversely effect fitness (Bauer 1992). Longer duration tests may help resolve this issue, although the ability to maintain juvenile mussels in a healthy condition in the laboratory over a longer exposure duration (i.e., > 2 months) is presently uncertain in water only exposures.

Water-only toxicity tests represent only one possible exposure route of synthetic musks to freshwater mussels. Mussels typically live buried in sediments and may feed on sediment-associated particles (Naimo 1995), thus, sediments may be an additional exposure route. Although data are limited, HHCB and AHTN were detected in surficial sediments from Lake Erie (4 ng HHCB/g and 0.5 ng AHTN/g) and Lake Ontario (19 ng HHCB/g and 1.2 ng AHTN/g) (Linebaugh 2004). Lastly, the potential for synthetic musk exposure to indirectly affect mussel populations through their host fish is unknown. Synthetic musks can impair estrogenic function in fish by suppressing the effects of 17β—estradiol on estrogen receptors in vitro and in vivo with transgenic fish (Schreurs et al. 2004).

Recently, HHCB and AHTN were found to compromise the multixenobiotic defense system in marine mussels, suggesting they may play a role as chemosensitizers that enable toxic multixenobiotic resistance substrates to accumulate in cells (Luckenbach and Epel 2005; Luckenbach et al. 2004). Given these recent mechanistic studies, endpoints that relate more directly to the mode of action of musks should be considered. Lethality is the most often used

endpoint in toxicological studies with unionids, although sublethal measures are being used more frequently (Naimo 1995; Newton et al. 2003). More research on sublethal indices of contaminant exposure (i.e., biomarkers associated with biotransformation enzymes, oxidative stress, and immunology) may help determine if musks are interfering with biological processes in freshwater mussels.

Conclusions

The present study provides data on the lethal and sublethal effects of polycyclic musks to glochidial and juvenile life stages in *L. cardium*. A dose-response relation was observed between two polycyclic musks and glochidia, with AHTN being toxic at lower concentrations than HHCB. Neither musk adversely affected the survival of juveniles, however, growth rates were significantly reduced upon exposure to either musk. Although concentrations tested in the laboratory are several orders of magnitude higher than ambient levels in the environment, these findings illustrate the potential for adverse sublethal effects of musk exposure to aquatic organisms.

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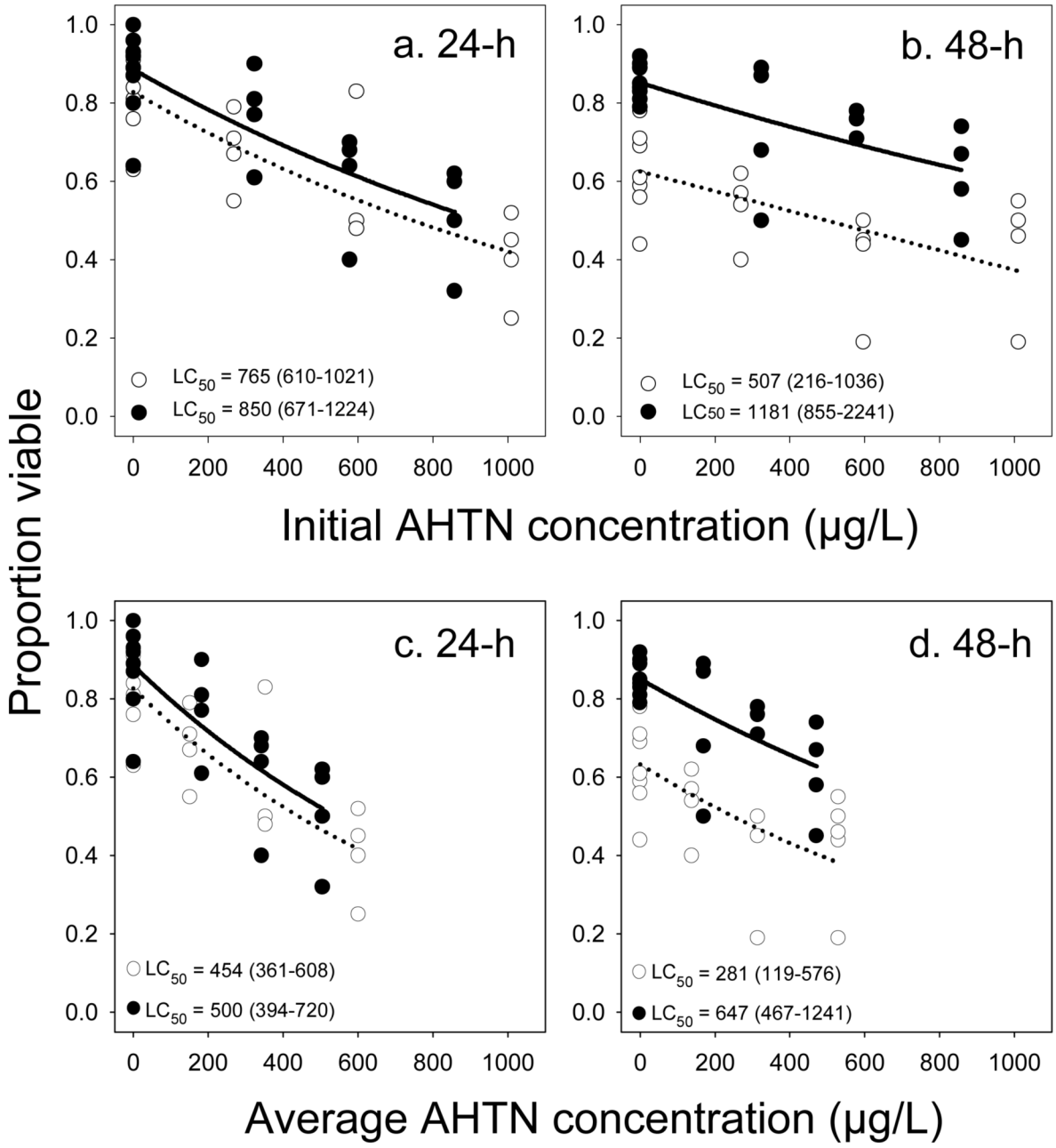

Fig. 1.

Median lethal concentrations (LC50, 95% confidence limits in parentheses) based on initial (a, b) and average (c, d) concentrations of AHTN during 24- and 48-h exposures of *Lampsilis cardium* glochidia. Average concentrations are the mean of the measured initial and final concentrations. Symbols are the actual data points (open and filled circles are replicate tests) and the lines are the model-predicted values.

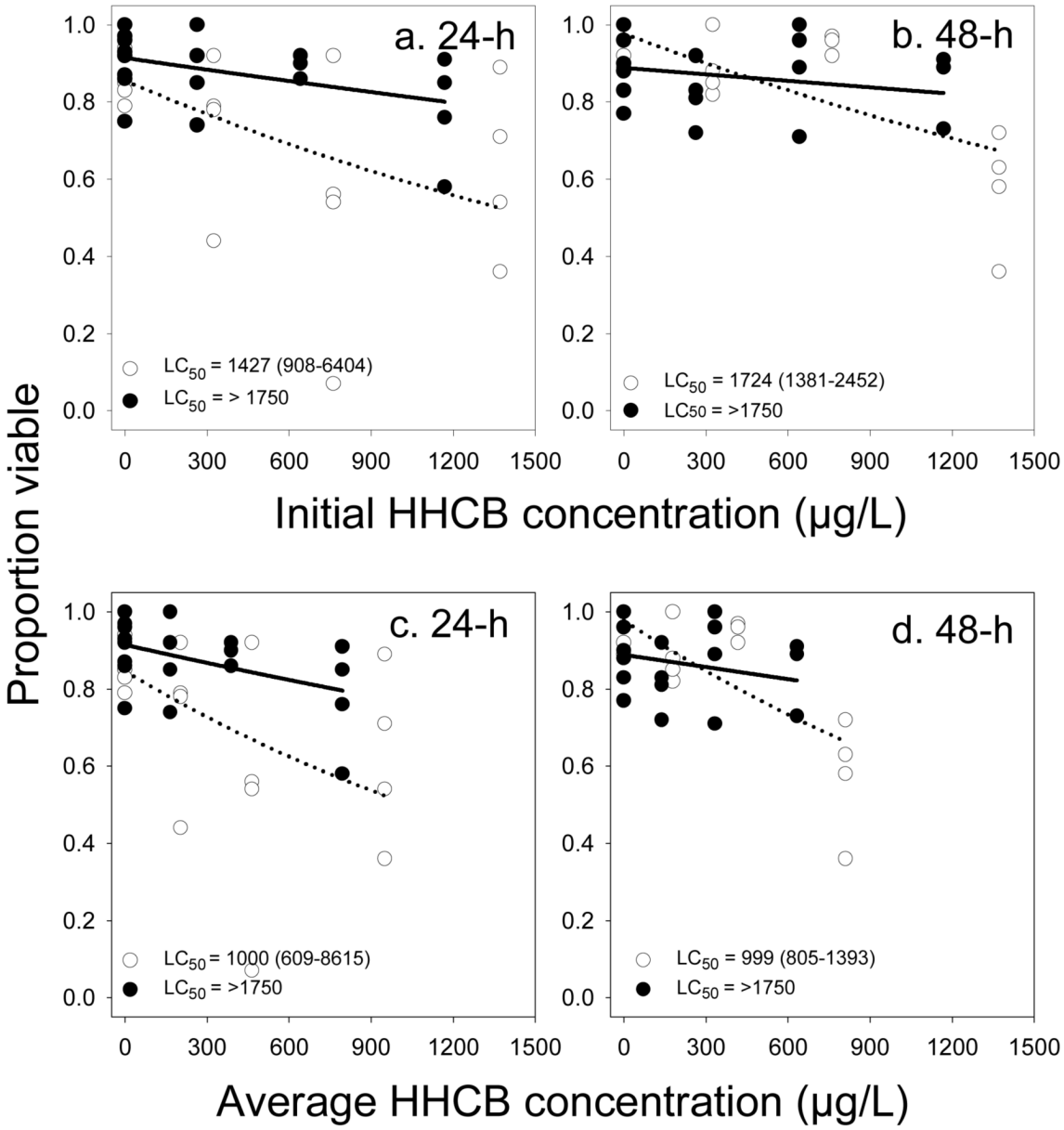

Fig. 2.

Median lethal concentrations (LC50, 95% confidence limits in parentheses) based on initial (a, b) and average (c, d) concentrations of HHCB during 24- and 48-h exposures of *Lampsilis cardium* glochidia. Average concentrations are the mean of the measured initial and final concentrations. Symbols are the actual data points (open and filled circles are replicate tests) and the lines are the model-predicted values. Note, all LC50s in the second test (filled circles) exceed the water solubility of 1750 µg/L.

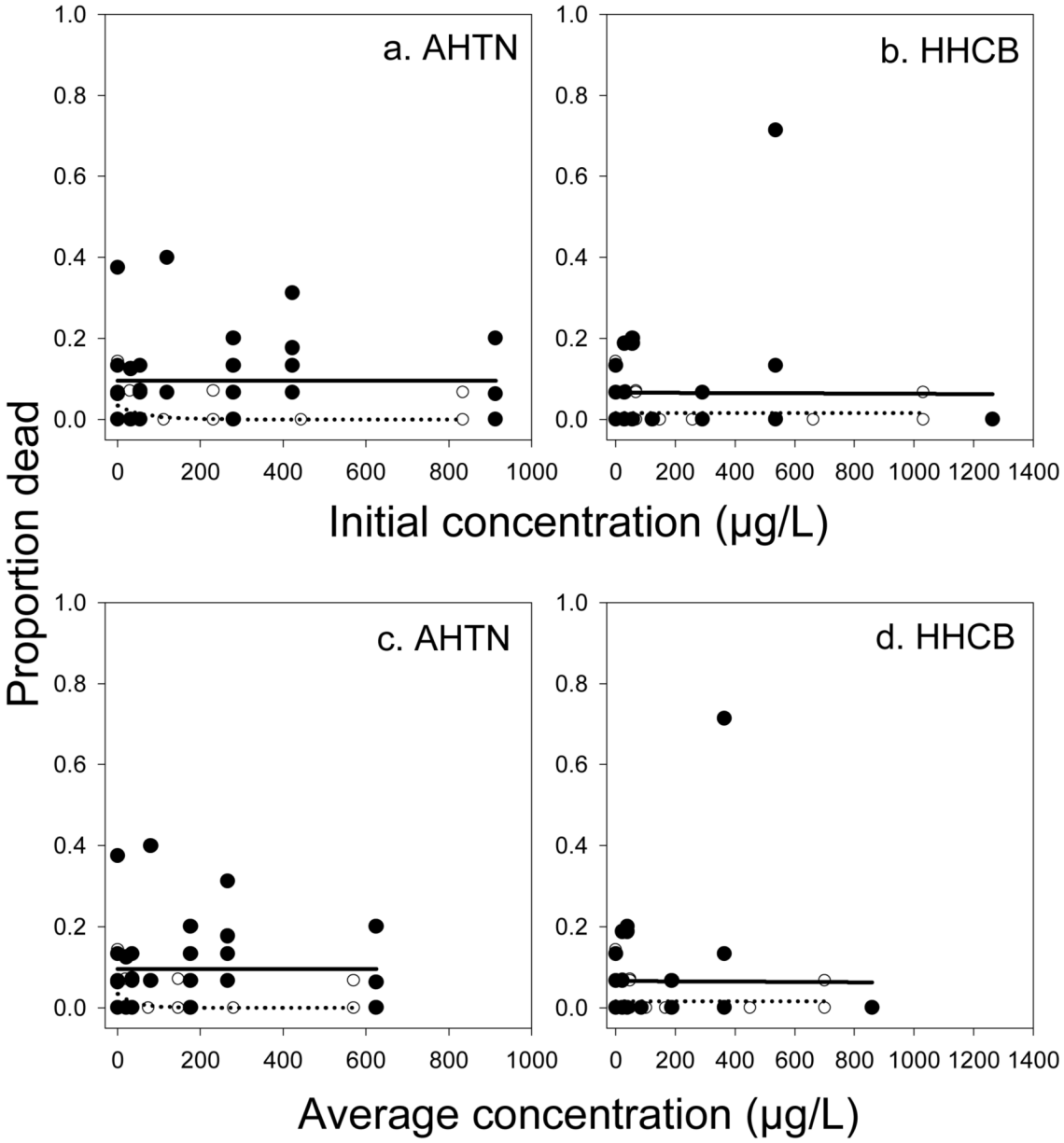

Fig. 3.

Mortality of juvenile *Lampsilis cardium* exposed to AHTN and HHCB during 96-h tests based on initial (a, b) and average (c, d) concentrations. Average concentrations are the mean of the measured initial and final concentrations. Symbols are the actual data points (open and filled circles are replicate tests), and the lines are the model-predicted values.

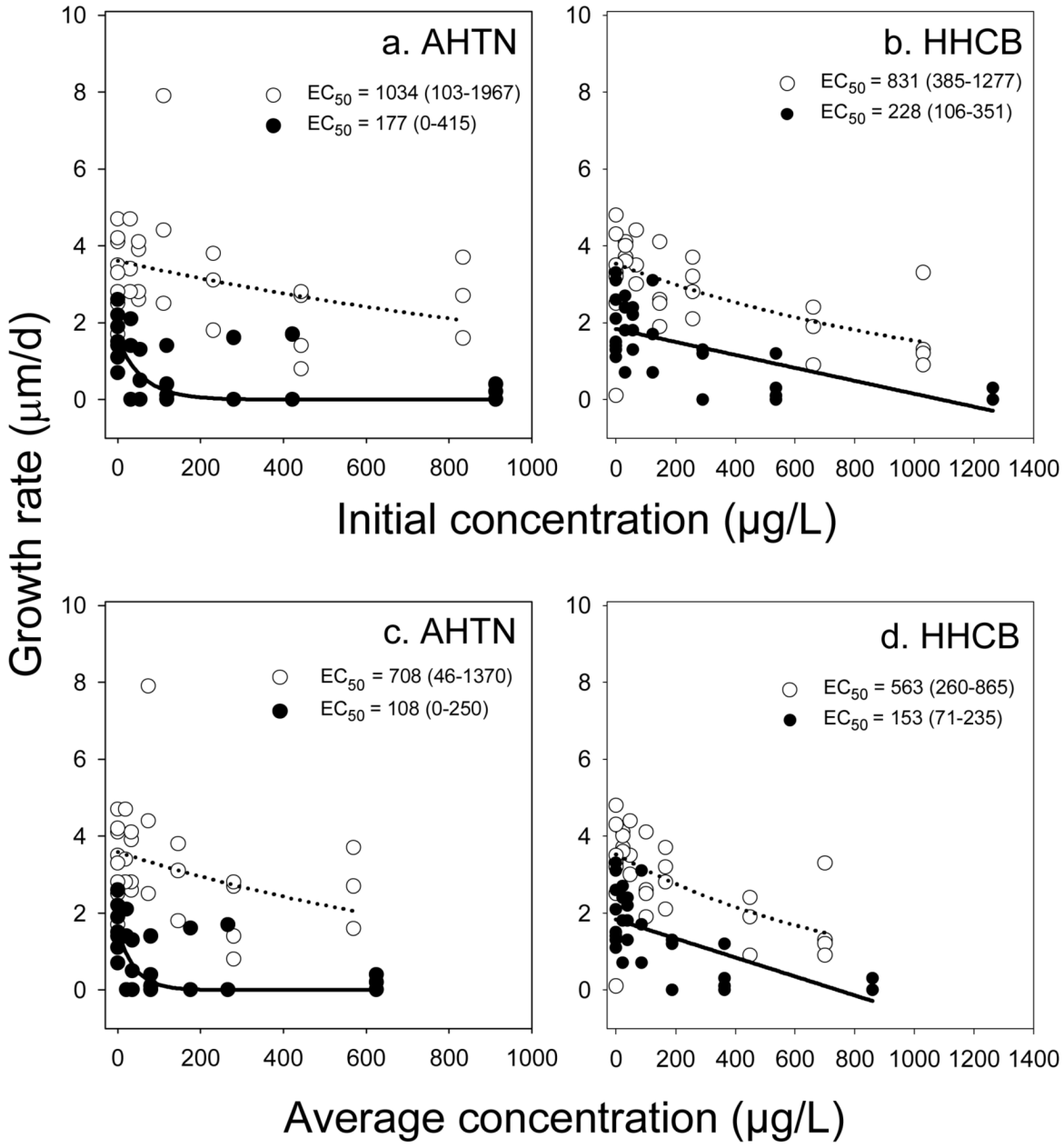

Fig. 4.

Median effective concentrations (EC50, 95% confidence limits in parentheses, based on shell height) as a function of initial (a, b) and average (c, d) concentrations of AHTN and HHCB during 96-h exposures with juvenile *Lampsilis cardium*. Average concentrations are the mean of the measured initial and final concentrations. Symbols are the actual data points (open and filled circles are replicate tests), and the lines are the model-predicted values.

Table 1

Initial and average measured concentrations (μg/L ± 1 SE) of AHTN and HHCB in replicate 24- and 48-h static tests with glochidia. The average concentration is the mean of the initial and final concentration in each test. Concentrations of musks in the well water and solvent controls were below detection limits

Chemical	Nominal (μg/L)	test 1 initial	24 h, test 1 average	48 h, test 1 average	test 2 initial	24 h, test 2 average	48 h, test 2 average
AHTN	300	269[a]	151[a]	138[a]	324 ± 5	182 ± 8	169 ± 7
AHTN	600	596 ± 7	352 ± 7	314 ± 7	578 ± 18	342 ± 18	314 ± 16
AHTN	1200	1010 ± 21	601 ± 22	529 ± 19	858 ± 4	505 ± 16	471 ± 6
HHCB	400	325 ± 4	204 ± 5	179 ± 5	264 ± 32	165 ± 35	138 ± 24
HHCB	800	762 ± 1	464 ± 10	417 ± 1	642 ± 32	388 ± 34	333 ± 34
HHCB	1600	1372 ± 29	950 ± 42	810 ± 15	1169 ± 14	795 ± 16	632 ± 40

[a]only one sample analyzed.

Table 2

Initial and average measured concentrations (μg/L ± 1 SE) of AHTN and HHCB in replicate 96-h tests with juveniles. The average concentration is the mean of the initial and final concentration in each test. Concentrations of musks in the well water and solvent controls were below detection limits

Chemical	Nominal (μg/L)	96 h, test 1 initial	96 h, test 1 Average	96 h, test 2 initial	96 h, test 2 average
AHTN	38	30 ± 4	19 ± 3	32 ± 5	20 ± 3
AHTN	75	51 ± 6	33 ± 4	54 ± 15	35 ± 4
AHTN	150	111 ± 10	74 ± 7	119 ± 14	79 ± 9
AHTN	300	231 ± 15	146 ± 10	280 ± 37	176 ± 24
AHTN	600	443 ± 25	280 ± 16	422 ± 54	251 ± 49
AHTN	1200	834 ± 56	570 ± 38	913 ± 97	623 ± 65
HHCB	50	32 ± 4	23 ± 3	30 ± 3	22 ± 2
HHCB	100	68 ± 9	48 ± 6	56 ± 5	38 ± 3
HHCB	200	147 ± 28	102 ± 20	123 ± 17	85 ± 12
HHCB	400	257 ± 14	166 ± 9	291 ± 32	185 ± 20
HHCB	800	662 ± 33	450 ± 28	536 ± 24	365 ± 26
HHCB	1600	1031 ± 49	701 ± 33	1264 ± 102	829 ± 73

Table 3

Percent of initial musk concentrations remaining in the highest concentration at 3, 6, 12 and 24 h after renewal of musk solutions during exposures with juvenile *Lampsilis cardium*

Chemical	Replicate	Percent remaining			
		3 h	6 h	12 h	24 h
AHTN	1	59	50	49	38
AHTN	2	54	55	47	37
AHTN	3	63	64	54	39
Mean	--	59	56	50	38
HHCB	1	61	53	43	36
HHCB	2	46	43	42	37
HHCB	3	48	47	33	31
Mean	--	52	48	39	35