Toxicity Comparison of the Shoreline Cleaners Accell Clean® and PES-51® in Two Life Stages of the Grass Shrimp, *Palaemonetes pugio*

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ABSTRACT

Oil spills are a significant source of coastal pollution. Shoreline cleaners, used to remove oil from surfaces during spill response and remediation, may also act as toxins. Adult and larval grass shrimp, *Palaemonetes pugio*, were tested for lethal and sublethal impacts from two shoreline cleaners, Accell Clean SWA® and PES-51®, alone and in combination with crude oil using Chemically Enhanced Water Accommodated Fractions (CEWAFs). Median lethal toxicity values determined for the individual cleaners were similar. However, when tested in mixture with oil as CEWAFs, Accell Clean SWA resulted in greater hydrocarbon concentrations in the water column and greater toxicity than PES-51. Increased glutathione levels were observed for adult shrimp exposed to Accell Clean SWA, and glutathione was elevated in shrimp exposed to both CEWAFs. Larval shrimp development was delayed after exposure to both CEWAFs. These findings may have implications for managing and mitigating oil spills.

Keywords: shoreline cleaner, oil, grass shrimp, Accell Clean SWA®, PES-51®, larval development

INTRODUCTION

Each year, oil spills and other anthropogenic sources, such as urban runoff and ship discharges, contribute to about five million metric tons of crude and refined oil pollution in the environment (Edwards et al., 2003; Johnston, 1984). Marine oil spills are detrimental to the environment as well as expensive to clean up. Approximately 80-90% of mitigation costs can be attributed to shoreline cleanup alone (Pereira and Mudge, 2004). Shoreline cleaning chemicals may be used to remove oil from solid surfaces such as beaches, seawalls, mangroves, and industrial equipment. The U.S. Environmental Protection Agency (USEPA) National Contingency Plan (NCP) serves as a national guide for oil spill and hazardous substance cleanup. The NCP lists 56 surface washing agents and 17 miscellaneous oil spill control agents for potential use on shorelines in the event of contamination (USEPA, 2017).

Shoreline cleaning agents remove oil from a surface by separating the oil from the substrate, by dispersing the oil in the water applied during cleaning, and/or by promoting degradation. Little is known about the environmental effects of these chemicals on estuarine organisms that may be impacted during oil spill remediation efforts. This study examined the toxicity of two of the compounds listed in the National Contingency Plan, Accell Clean SWA® and PES-51®, to the estuarine grass shrimp, *Palaemonetes pugio*.

The estuarine grass shrimp, *Palaemonetes pugio*, is a well-studied crustacean species used as a bioindicator of anthropogenic impacts (Key et al., 2006) and as common toxicity test species (Buikema et al., 1980). *P. pugio* is widely distributed in the western Atlantic and Gulf of Mexico (Kaplan, 1988), where they act as primary and secondary consumers, and aid in the breakdown of detritus (Key et al., 2006). Many recreationally and commercially-valuable fish and crab species use estuaries as nursery grounds and prey on *P. pugio* (Welsh, 1975). There are

four life stages during the life cycle of *P. pugio*: embryo, larvae, juvenile (postlarvae), and adult (Manyin and Rowe, 2010). Grass shrimp mature at around 1.5 to 2 months old and reach an adult length of about 15 to 18 mm (Anderson, 1985). Their life span is 6 to 13 months (Alon and Stancyk, 1982).

The goal of this project was to evaluate the toxicity of Accell Clean SWA and PES-51 alone and in combination with Louisiana Sweet Crude (LSC) oil in the grass shrimp, *P. pugio*. The first objective of this project was to determine 96-h median lethal concentration (LC_{50}) values for all four treatments for two life stages (adult and larvae), and to compare the results. The second objective was to measure sublethal effects of the shoreline cleaners, including glutathione levels as a cellular stress biomarker in adult shrimp, ecdysteroid molting hormone levels in larval shrimp, and the subsequent development of larval grass shrimp after acute exposures. This work provides essential information for evaluating toxicity of two common shoreline cleaners on an important estuarine species, using both lethal and sublethal endpoints.

MATERIALS AND METHODS

Test Species Collection and Holding

Non-ovigerous (i.e. not egg-bearing) adult grass shrimp (approximately 15-18 mm in length) were collected from Leadenwah Creek (N 32° 38’ 51.00”; W 80° 13’ 18.05”). Seawater was acquired from Charleston Harbor estuary (N 32° 45’ 11.52”; W 79° 53’ 58.31”), pre-filtered (5 μm), activated carbon filtered, and diluted with deionized water to adjust salinity to 20 ppt. Shrimp were acclimated for 7-14 days in 76 L tanks at 25°C, 20 ppt salinity, and 16-h light: 8-h dark photoperiod. While acclimating, shrimp were fed Tetramin® fish flakes daily. To obtain larval grass shrimp, ovigerous females were collected and acclimated as previously described.

Ovigerous females were then placed in brooding traps to allow larvae (zoea) to hatch and escape without interference. Larvae were pooled from at least 10 females. Grass shrimp larvae were fed cultured *Artemia* nauplii after hatching and were tested at 24-48h old.

Shoreline Cleaner Products

Accell Clean® SWA was obtained from Advanced BioCatalytics, Irvine, CA, USA. Information from the manufacturer states that it is a combination of commonly used surfactants with non-enzymatic proteins from baker's yeast. The protein-surfactant complexes are designed to stimulate bacterial oil consumption without increasing bacterial biomass. PES-51® was obtained from Practical Environmental Solutions, San Antonio, TX, USA. PES-51 consists primarily of d-limonene, a terpene chemical produced naturally by citrus plants and some coniferous trees. According to the manufacturer, PES-51 is also composed of bacterial fermentation by-products that, in combination with the carrier solvent, d-limonene, form a "unique biological mixture" that surrounds hydrocarbon molecules and lifts them from surfaces (Hoff et al., 1994). Specific chemical ingredients of both products are considered proprietary.

Shoreline Cleaner Acute Toxicity Tests

Acute 96-h static renewal tests were performed in an environmental chamber at 25°C and a 16-h light:8-h dark photoperiod. Adult shrimp were exposed in 4 L wide-mouth glass jars containing 2 L of aerated 20 ppt seawater with 10 shrimp/jar and three replicates/treatment. Larval shrimp (24-48 h old) were exposed in 600-mL glass beakers containing 400 mL of aerated 20 ppt seawater with 10 larvae/beaker and three replicates/treatment. Treatment concentrations were determined from a preliminary range finding test. Nominal Accell Clean SWA concentrations for both life stages and PES-51 concentrations for adults were 4.1, 12.3, 37, 111, and 333 mg/L. Nominal PES-51 concentrations for larval aqueous exposures were 12.3, 37,

111, 333, and 1000 mg/L. A seawater control was included for each assay. Every 24 h, mortality was assessed, dead shrimp were removed, and test solutions were renewed. Water quality parameters (dissolved oxygen, pH, temperature, and salinity) were measured prior to each 24-h renewal from one replicate jar per treatment.

Shoreline Cleaner and Oil (CEWAF) Acute Toxicity Tests

The shoreline cleaner with oil exposure was prepared using Chemically Enhanced Water Accommodated Fractions (CEWAFs) of the shoreline cleaners in mixture with Louisiana Sweet Crude (LSC) oil. Preparation of the CEWAFs followed methods similar to Hemmer et al. (2011) and DeLorenzo et al. (2017). A clean glass aspirator bottle was placed on a stir plate and the bottom outlet closed with Tygon tubing and a glass stopper. A Teflon stir bar was placed in the bottom of the aspirator bottle. Seawater (18 L, 20 ppt) was added to the aspirator bottle and stirring was initiated. Next, 25 g/L of oil was added to the center of the vortex using a graduated cylinder. The initial weight and weight after dispensing were recorded to determine the actual amount added by mass difference. The cleaner was then added to the center of the vortex using a glass pipette at a ratio of 1:10 shoreline cleaner:oil (or 2.5 g shoreline cleaner/L), and again delivery mass was calculated by difference in weight. The aspirator bottle was then sealed with a stopper, the mixing speed increased to achieve a vortex 25% of the solution height, and the solution stirred for 18 h. After letting the solution sit for 6 h, the stopper was removed, the bottom outlet opened, and the CEWAF dispensed into a collection container, without disturbing the oil slick layer. The CEWAFs were prepared in the dark and used immediately. The 100% CEWAF was diluted with 20 ppt seawater to achieve additional treatments (50%, 16.7%, 5.6%, 1.9%, 0.6%, and 0.2%). Each test included a seawater control. Similar test methods were used as for the shoreline cleaner alone testing except the CEWAF solutions was not renewed. Water

quality parameters (dissolved oxygen, pH, temperature, and salinity) were measured from one replicate jar per treatment at the end of the 96 h exposure.

Chemical Analysis

The shoreline cleaners were not quantified chemically given the proprietary nature of the products. Shoreline cleaner-CEWAF water samples (1000 mL) were collected for analysis of total extractable hydrocarbons (TEHs) and polycyclic aromatic hydrocarbons (PAHs) immediately after dosing for each CEWAF concentration and a 20 ppt seawater control using methods from DeLorenzo et al. (2017). Briefly, samples were acidified to a pH of 2 and then transferred into solvent-rinsed 1-L separatory funnels to undergo liquid/liquid extraction. Samples were spiked with isotopically labeled internal standards and then solvent extracted three times with the following solvents, dichloromethane, 50:50 dichloromethane/hexane, and hexane. After extraction, samples were passed through GF/F paper containing anhydrous sodium sulfate and concentrated in a water bath (40°C) under a stream of nitrogen (14 psi). Extracts were prepared using silica SPE and spiked with a recovery standard prior to instrumental analysis on GC/MS.

Extracts were run on an Agilent 6890/5793N GC/MS with split/splitless injector containing a DB17ms analytical column (60m x 0.25 mm x 0.25µm). The mass spectrometer was operated in selected ion monitoring (SIM) mode. Two separate instrumental runs were required to acquire the PAH and TEH data. A total of 50 PAHs were analyzed, including both parent and alkylated PAHs (Appendix 1). TEH was quantified by integrating the chromatogram from C9-C44 based on the response from ion 57. Data analysis was performed using MSD Chemstation software.

Sublethal Assessments

Glutathione

Surviving adult grass shrimp were frozen at -80°C after each toxicity test and analyzed for glutathione. Glutathione was assessed using the 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB)-glutathione reductase recycling protocol described in Hoguet and Key (2007). Frozen shrimp (1-9 samples per replicate) were weighed, homogenized cold in 5% sulfosalicyclic acid (SSA), and centrifuged cold (4°C) for 5 min at 13,000 g. A total of 975 µL of a mixture of DI water, 5,5'-dithiobis (2-nitrobenzoic) acid (DTNB), and β-nicotinamide adenine dinucleotide phosphate (NADPH), reduced form, Sigma-Aldrich) buffer were added to 25 µL sample supernatant. Glutathione standards (Sigma-Aldrich) were dissolved in SSA and 25 µL of each concentration (200, 100, 50, 25, 12.5, and 6.25 µM) were added to the previously described mixture. The blank contained only SSA. Fifty units/ ml glutathione disulfide (GSSG) reductase (from Baker's yeast, Sigma-Aldrich) was added to the samples and standards and placed in a spectrophotometer. Absorbance was read at 405 nm for 90 s with 15 s intervals. Data were expressed as nM of glutathione formed per gram of wet weight.

Ecdysteroid ELISA

For the measurement of molting hormone levels in larval shrimp after 96 h and assessment of subsequent larval development, tests were conducted similarly to those described above for each shoreline cleaner and oil mixture. Nominal shoreline cleaner concentrations were selected based on the results of the definitive 96-h tests (Accell Clean SWA: 4.1, 12.3, and 37 mg/L; PES-51: 12.3, 37, and 111 mg/L; Accell Clean-CEWAF: 0.2%, 0.6%, 1.9%, and 5.6%; PES-51-CEWAF: 0.6%, 1.9%, 5.6%, 16.7%, 50%, and 100%). There were three replicate beakers per treatment with ten larvae per beaker, along with at least three replicate 6-well plates

per treatment with one larva per well. The three beakers per treatment were terminated after 96 h and surviving larvae were frozen at -80°C for ecdysteroid analysis.

A modified ecdysteroid ELISA protocol was used to assess larval shrimp ecdysteroid activity after 96-h exposure (Cayman Chemical, 2009; Gelman et al., 2002; Tuberty and McKenney, 2005). Larval shrimp, 7-10 individuals depending on availability, were weighed, homogenized on ice in 80% methanol (50 µL/shrimp), and centrifuged at 14,000 g for 5 min at 4°C. The supernatant was transferred to new tubes and placed on ice. An additional 50 µL/shrimp of 80% methanol was added to the precipitates, homogenized for 1 minute, and centrifuged again at 14,000 g for 5 min at 4°C. The second supernatant was added to the corresponding first supernatant on ice. The methanol was evaporated under nitrogen in a TurboVap® LV (Caliper Life Sciences). The sample was reconstituted by adding 50 µL/shrimp of EIA buffer (Cayman Chemical) to each sample tube and vortexed. One hundred µL of EIA buffer was added to the non-specific binding (NSB) wells and 50 µL to the maximum binding (B0) wells in a 96-well plate (Cayman Chemical). Fifty µL of standards (32, 16, 8, 4, 1, 0.2, 0.1, and 0.02 Fmol/µL) and samples were added to the appropriate wells. Tracer (50 µL) was added to all wells except the blank (Blk) and total activity (TA) well and antiserum (50 µL) was added to all wells except Blk, TA, and NSB. The plate was covered with plastic film and incubated overnight (18 h) at 4°C. Contents were discarded and wells were washed with wash buffer five times. Ellman's reagent (DTNB, 200 µL) was added to each well and tracer (5 µL) was added to the TA well. The plate was developed in the dark for 90 min. Absorbance was read in a Bio-tek Instruments µQuant microplate spectrophotometer at a wavelength of 418 nm.

Developmental Bioassay

Larvae from the remaining 6-well plates were moved to new clean plates containing clean seawater and post-exposure larval development was assessed. Each day, molts were counted and removed and larval developmental status was observed. On Mondays, Wednesdays, and Fridays water quality (temperature, salinity, pH, and dissolved oxygen) was measured, the well plates were renewed with clean 20 ppt seawater, and the larvae were fed 50 µL of *Artemia*. The test was terminated when larvae in all concentrations reached post-larval status. *P. pugio* larvae were characterized as swimming upside down and backward and containing pairs of chromatophores (Key et al. 1998). Post-larval status was characterized as swimming right-side up and forward after the final larval molt and loss of the chromatophore pairs. Surviving larvae that reached post-larval status were oven dried for 48 h at 60°C to determine dry weight (Key and Fulton, 1993).

Statistical Analyses

Median Lethal Concentration (96-h LC_{50}) values with 95% confidence intervals (CIs) were determined using probit analysis (SAS Probit Analysis, SAS V.9.4, Cary, NC). The test concentrations for the model were based on nominal shoreline cleaner chemical concentrations for the individual chemicals and measured TEH and PAH concentrations for the CEWAFs. LC_{50} ratio tests (SAS LC_{50} Ratio Test, SAS V.9.4, Cary, NC) were used to determine significant differences ($p < 0.05$) between the different life stage and shoreline cleaner LC_{50} values (Wheeler et al., 2006). Analysis of Variance (ANOVA) tests were conducted to determine significant differences ($p < 0.05$) among treatments for the following measurements: survival, glutathione, dry weight, number of larval instars, time to post-larval stage, and ecdysteroid levels. Dunnett's tests for multiple comparisons were used to identify which concentrations were significantly different from the control. In cases where data did not meet the assumptions for ANOVA, a

Kruskall-Wallis nonparametric test was performed. Where ANOVA p-values were significant, but Dunnett's test did not show significant difference from control, a William's Monotonic Trend test was conducted to determine if the trend was significant and to identify the lowest observable effect concentration (LOEC).

RESULTS

Water quality for all toxicity tests was maintained within acceptable ranges for dissolved oxygen (≥ 60% saturation), pH (8.0 ± 0.5), temperature (25 °C ± 2), and salinity (20 ppt ± 2). Control survival for all definitive tests met protocol standards (≥ 90%).

Shoreline Cleaner Alone

Accell Clean SWA concentrations ≥37 mg/L resulted in significant mortality compared to control for both adult and larval grass shrimp (ANOVA p values for each experiment <0.0001) (Figure 1). Exposure of grass shrimp to Accell Clean SWA resulted in 96-h aqueous LC_{50} values of 44.18 mg/L (95% CI: 30.39-60.52) for adults and 48.64 mg/L (95% CI: 41.62-80.62) for larvae, and the toxicity values for the adult and larval life stages were not significantly different (LC50 ratio p=0.0962) (Table 1).

For PES-51 adult grass shrimp were significantly more sensitive than larval shrimp (LC50 ratio p<0.0001), with a 96-h LC_{50} value of 38.75 mg/L (95% CI: 17.99-65.43) compared to 155.42 mg/L (95% CI: 127.43-200.28) for larvae (Table 1). PES-51 concentrations ≥37 mg/L resulted in significant mortality of adult shrimp, whereas concentrations ≥111 mg/L were significantly different from control for larval grass shrimp (ANOVA p values for each experiment <0.0001) (Figure 1). Adult grass shrimp mortality was 73.3% for 37 mg/L PES-51 compared to 1.7% mortality for larval shrimp (Figure 1). Larval grass shrimp were significantly

more tolerant to PES-51 than Accell Clean SWA (LC50 ratio p<0.0001) (Table 1). Larval mortality was 33.3% at 111 mg/L PES-51 compared to 100% mortality for larvae exposed to 111 mg/L Accell Clean SWA (Figure 1). There was no significant difference between adult LC_{50} values for Accell Clean SWA and PES-51 (LC50 ratio p=0.1311) (Table 1).

Shoreline Cleaner and Oil CEWAF

Accell Clean CEWAF concentrations ≥16.7% resulted in significant mortality compared to control for both adult and larval grass shrimp (ANOVA p values for each experiment <0.0001) (Figure 2). PES-51 CEWAF concentrations ≥50% resulted in significant mortality compared to control for both adult and larval grass shrimp (PES-51 adult ANOVA p=0.0029; PES-51 larvae ANOVA p=0.0056) (Figure 2). The Accell Clean CEWAF was significantly more toxic to both life stages of the grass shrimp than the PES-51 CEWAF. Both adult and larval grass shrimp mortality at 50% PES-51 CEWAF was 3.3% compared to 100% for the Accell Clean CEWAF (Figure 2). LC_{50} values could not be determined for the PES-51 CEWAF because less than 50% mortality occurred in the full-strength CEWAF. Adult grass shrimp mortality at 16.7% Accell Clean CEWAF was 33.3% compared to 100% mortality at the same percent CEWAF concentration for larval shrimp (Figure 2).

TEH and total PAH concentrations were higher in the Accell Clean CEWAF compared to the PES-51 CEWAF (Table 2). TEH concentrations for the PES-51 CEWAF decreased from 7.6 mg/L in the 100% CEWAF solution to less than 0.25 mg/L (the detection limit) for 5.6%, 1.9%, 0.6% and the control treatments (Table 2). TEH concentrations for the Accell Clean CEWAF ranged from 72 mg/L (100% CEWAF) to less than detection (<0.25 mg/L) for 0.2% and the control treatments (Table 2). Total PAH concentrations for the Accell Clean CEWAF decreased from 951.08 µg/L (100% CEWAF) to 2.56 µg/L for 0.2% CEWAF treatment and below detection

for the control (Table 2). Total PAH concentrations for the PES-51 CEWAF decreased from 528.50 µg/L (100% CEWAF) to 5.17 µg/L for the 0.6% CEWAF treatment.

Accell Clean CEWAF LC_{50} values determined using measured TEH concentrations were 1.86 mg/L (95% CI: 1.51-3.86) for adult shrimp and 1.14 mg/L (95% CI: 1.01-1.28) for larvae; with adults being more tolerant than larvae (LC50 ratio p=0.0476) (Table 3). Accell Clean CEWAF LC_{50} values determined using measured total PAH concentrations were 113.99 µg/L for adult shrimp (95% CI: 98.98-247.97) and 80.61 µg/L (95% CI: 33.13-106.76) for larvae; with adults being significantly more tolerant than larvae (LC50 ratio p=0.0015) (Table 3). LC_{50} values could not be determined for the PES-51 CEWAF because for both life stages, less than 50% mortality occurred in the full-strength CEWAF. Based on the measured chemistry, however, the adult and larval 96-h LC_{50} values can be reported as >7.6 mg/L TEH (Table 3) and >528.50 µg/L total PAH (Table 3).

Sublethal Assessments

Glutathione Assay

Glutathione levels were significantly higher at 37 and 111 mg/L Accell Clean (37 mg/L: 632.68 nmol/g wet weight; 111 mg/L: 602.44 nmol/g wet weight) compared to the control (257.34 nmol/g wet weight) (p=0.0004) (Table 4). There were no significant differences found between PES-51 treatments and controls (p=0.8366) (Table 4).

In both shoreline cleaner CEWAF exposures, a significant difference in glutathione levels was measured (Accell Clean CEWAF: p=0.0028; PES-51 CEWAF: p=0.0004). Adult grass shrimp glutathione levels were significantly elevated compared to control levels in the 16.7% Accell Clean CEWAF and in the 100% PES-51 CEWAF (Table 4).

Ecdysteroid ELISA Assay

After acute 96 h exposure to Accell Clean SWA, ecdysteroid concentrations in larval grass shrimp were elevated significantly (24 times greater) in the highest treatment group (37 mg/L) when compared with the control level (p=0.0105) (Table 5). No significant differences in ecdysteroid levels were found between PES-51 treatment groups (p=0.2772) (Table 5).

Ecdysteroid levels were significantly different between Accell Clean CEWAF treatments (ANOVA p=0.0080). Hormone levels increased from 98.9 $x10^3$ ng 20-HE/g wet weight in the 0.6% CEWAF treatment to 203 $x10^3$ ng 20-HE/g wet weight in the 5.6% Accell Clean CEWAF (Table 5). A William's test for monotonic trend determined the lowest observable effect concentration for increasing ecdysteroid level in the Accell Clean CEWAF was 5.6% (p=0.0426). No significant differences in ecdysteroid levels were found between PES-51 CEWAF treatment groups (p=0.1755) (Table 5).

Development Measurements

Dry Weight

The mean dry weight of larvae at post-larval status ranged from 707.7 µg (control) to 807.4 µg (12.3 mg/L) for shrimp exposed to Accell Clean (Table 6). Larval shrimp exposed to PES-51 mean dry weights ranged from 811.8 µg at 37 mg/L to 920 µg at 333 mg/L (Table 6). There were no significant dry weight differences from the control for either Accell Clean or PES-51 (Accell Clean: p=0.1056; PES-51: p=0.2801).

The mean dry weight of larvae at post-larval status differed significantly among Accell Clean CEWAF treatments (p=0.0037). The mean dry weight was heaviest for the 5.6% Accell Clean CEWAF group at 858.2 µg, while the mean dry weight for the control was 745.4 µg (Table 7). The mean dry weights were similar between PES-51 CEWAF concentrations, ranging from

727.4 µg at 5.6% CEWAF to 784.5 µg at 100% CEWAF (Table 7). There were no significant difference in dry weight among PES-51 CEWAF treatments (p=0.6339).

Days to Postlarvae

The mean number of days for development per larva to reach post-larval status differed among Accell Clean treatments (p=0.0464). The 12.3 mg/L group took the least number of days to reach postlarvae (14.9 days) while the control took the longest (16.4 days) (Table 6). The mean number of days to post-larval development ranged from 18.8 and 18.8 days for the control and 12.3 mg/L PES-51 respectively, to 22.0 days for 333 mg/L PES-51 (Table 6). However, there was no significant difference in number of days to post-larval stage among PES-51 treatments (p=0.0807).

The mean number of days for development per larva to reach post-larval status differed significantly among treatments for both the Accell Clean and PES-51 CEWAFs (p-values <0.0001). For the Accell Clean CEWAF, the 5.6% CEWAF group took the longest time to reach postlarvae at 18.9 days, compared with the control at 16.6 days (Table 7). The longest time to reach postlarvae for the PES-51 CEWAF was 27.8 days at 5.6% CEWAF, compared with the control at 22.6 days (Table 7).

Number of Molts

The mean number of molts for grass shrimp larvae followed similar trends to the number of days for larvae to reach post-larval status. The difference in mean number of molts per larva to reach post-larval status differed significantly among Accell Clean SWA treatments (p=0.0179). The 12.3 mg/L group was lowest at a mean of 5.8 molts while the control was highest with a mean of 7.1 molts (Table 6). The mean number of molts increased from 8 molts at 12.3 mg/L to

9 molts at 111 mg/L PES-51 but there were no significant differences among PES-51 treatments (p=0.4148) (Table 6).

The mean number of molts per larva to reach post-larval status differed significantly among treatments for both the Accell Clean and PES-51 CEWAFs (Accell Clean CEWAF: p=0.0022; PES-51 CEWAF: p=0.0033). For the Accell Clean CEWAF, the 5.6% CEWAF had the most molts (mean of 7.7 molts) while the control had the lowest (mean of 6.4 molts) (Table 7). The 100% PES-51 CEWAF had the highest number of molts (mean of 7.2 molts), while the control had the lowest (mean of 5.9 molts) (Table 7).

DISCUSSION

Shoreline cleaners can be valuable tools for oil spill mitigation, and understanding the potential toxic effects on coastal species is key to their appropriate use. The results of this study generated new toxicity thresholds for two shoreline cleaners in a common estuarine crustacean species, the grass shrimp, *Palaemonetes pugio*. Differences in sensitivity by shoreline cleaner product and grass shrimp life stage were noted, and sublethal effects on shrimp physiology and development were quantified.

Shoreline Cleaner Toxicity

Few ecotoxicity values were available for Accell Clean SWA and PES-51 before this study. Values determined previously for Accell Clean SWA, including a 48-h LC_{50} value of 59.46 mg/L for an estuarine crustacean, *Mysidopsis bahia* (USEPA, 2011) and the 96-h LC_{50} values for Accell Clean SWA with adult and larval *P. pugio* obtained in this study, were similar (Adult: LC_{50} = 44.18 mg/L; Larvae: LC_{50} = 48.64 mg/L), although the lengths of exposure differed.

Toxicity values available in the literature for PES-51 include a 48-h LC_{50} value of 54 mg/L for *M. bahia* and a 96-h LC_{50} value of 137 mg/L for *Menidia beryllina* (USEPA, 2011). Adult grass shrimp in this study had a comparable LC_{50} value (LC_{50} = 38.75 mg/L) to *M. bahia* while larval grass shrimp from this study had a higher LC_{50} value (LC_{50} = 155.42 mg/L) than the adults and were similar in sensitivity to *M. beryllina*. Although larval grass shrimp are typically more sensitive to contaminants than adult shrimp (DeLorenzo et al., 2006; DeLorenzo et al., 2016; DeLorenzo and DeLeon 2010; Key et al., 1998; Key et al., 2003a; Key et al., 2005), occasionally adult grass shrimp have been shown to be more sensitive than larvae, such as when exposed to fipronil and endosulfan (Key et al., 2003b).

While Accell Clean SWA and PES-51 were similar in toxicity with the exception of larval shrimp being significantly less sensitive to PES-51, when the shoreline cleaners were mixed with LSC oil as a CEWAF, Accell Clean SWA was significantly more toxic to both life stages of grass shrimp compared to PES-51. Furthermore, larval grass shrimp were significantly more sensitive to the Accell Clean CEWAF compared to adults. Larval shrimp are growing at a faster rate than adult shrimp and generally have a higher metabolic rate than adult shrimp (DeLorenzo et al., 2006). This could lead to increased uptake of contaminants and increased sensitivity. LC_{50} values could not be determined for the PES-51 CEWAF with either life stage because there was less than 50% mortality in the 100% CEWAF. On the other hand, the Accell Clean CEWAF had 100% mortality at 50% and 100% CEWAF concentrations for adult shrimp and at all concentrations ≥16.7% CEWAF for larval shrimp. Since grass shrimp toxicity was similar for Accell Clean SWA and PES-51 when tested as individual products, our findings suggest that the difference in product toxicity seen with the CEWAFs is a result of differences in how these two shoreline cleaners interact with oil.

Shoreline Cleaner Chemistry

The Accell Clean CEWAF appeared to disperse the oil into the water column, while the PES-51 CEWAF appeared to have the oil and cleaner settle as a slick on the surface. The total PAH concentration for the 100% Accell Clean CEWAF was 951.08 µg/L, almost 2 times greater than the 100% PES-51 CEWAF concentration (528.20 µg/L). The PES-51 CEWAF contained a similar level of total PAH as a LSC oil WAF with no chemicals added (496.40 µg/L) (unpublished data). When LC_{50} values were expressed as TEH and total PAH in the CEWAFs, the Accell Clean CEWAF was significantly more toxic than the PES-51 CEWAF, which is counter to what we would expect if total hydrocarbons were driving the toxicity. If all the toxicity in the CEWAF was from total hydrocarbon exposure and the products were of equal toxicity, then the LC_{50} values would be similar because the same amount of oil was added to both CEWAFs. A 96-h LC50 value of 210.03 µg/L total PAH was previously determined with larval *P. pugio* exposed to LSC oil as a WAF (DeLorenzo et al., unpublished data), which suggests that Accell Clean-CEWAF (96-h LC50 = 80 µg/L total PAH) is more toxic than oil alone and that PES-CEWAF (96-h LC50 >528 µg/L total PAH) is less toxic than oil alone. CEWAFs are complex chemical mixtures of both shoreline cleaner constituents and oil constituents. We can speculate that the increased toxicity of the Accell Clean CEWAF is due to either changes in the dissolved fractions of the product or changes in dissolved fractions of hydrocarbons. This highlights the need for consideration of both the toxicities of the products alone and when mixed with oil because different shoreline cleaners elicit different chemical interactions with oil that will affect bioavailability and toxicity to aquatic species.

Sublethal Assessments

Sublethal effects at a cellular level for adult grass shrimp were detected by examining glutathione levels in response to Accell Clean and PES-51 alone as well as in combination with oil. Oxidative stress from shoreline cleaners mixed with oil as CEWAFs was observed to increase glutathione levels as an antioxidant response.

Larval shrimp were examined for molting hormone levels after acute exposure to the shoreline cleaners and the oil-cleaner mixtures, and for subsequent developmental effects. No significant relationship for the molting hormone ecdysteroid was established with either PES-51 alone or the PES-51 CEWAF. Larval shrimp had significantly higher ecdysteroid levels when exposed to Accell Clean SWA and the Accell Clean CEWAF. Tuberty and McKenney (2005) also reported increased ecdysteroid levels with pesticide exposure in larval grass shrimp that were approximately the same stage of development (instar 3) as the five to six-day-old shrimp assessed in this study, but pesticide exposure was associated with decreased molting hormone at later developmental stages. Changes in ecdysteroid concentrations may indicate endocrine disruption in crustaceans and can have long-term effects on growth, development, and reproduction of the organism (Lafontaine et al., 2016).

There were no significant differences in grass shrimp mean dry weight at post-larval status, number of days to post larva, or number of molts until postlarvae for larval shrimp exposed to PES-51 for 96 hours. Mean dry weight of grass shrimp at post-larval status was also not significantly affected with exposure to Accell Clean SWA, while mean number of days to postlarva and mean number of molts to post-larval status were significantly lower than the control only in the 12.3 mg/L treatment. As there was not a dose-response relationship with molting and duration of larval stage, we conclude that the shoreline cleaners alone would not have an impact on grass shrimp development.

Larvae exposed to the 5.6% Accell Clean CEWAF had significantly higher mean dry weight, number of days to post-larval status, and number of molts compared to the control. Similarly, larvae exposed to the 100% PES-51 CEWAF had significantly higher duration of development and number of molts compared to the control, as well as the highest mean dry weight. This suggests that shoreline cleaners mixed with oil may delay grass shrimp development, and potentially lead to changes in the population composition. Future studies should examine ecdysteroid levels at different stages of larval development in order to understand the relationship between hormone levels and duration of larval development, and ultimately how these measures relate to grass shrimp population success.

Salt marsh ecosystems are sensitive habitats that may be susceptible to oil and to oil spill mitigation chemicals used during clean up. Accell Clean SWA prepared as a chemically-enhanced water accommodated fraction (CEWAF) with LSC oil was observed to disperse/mix oil into solution, thus yielding greater concentrations of soluble hydrocarbons than PES-51 prepared as a CEWAF. The Accell Clean CEWAF was significantly more toxic to both life stages of grass shrimp compared to the PES-51 CEWAF. Larval shrimp were more sensitive to the Accell Clean CEWAF than adults and effects on larval growth and development were observed. This new information on shoreline cleaner product toxicity and chemical interactions with oil will allow managers to make more informed oil spill mitigation decisions.

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Table 1: Summary of 96-h LC_{50} values (and corresponding 95% confidence intervals) for adult and larval grass shrimp, *Palaemonetes pugio*, to two shoreline cleaners, Accell Clean and PES-51. Toxicity values were calculated using nominal cleaner concentrations (mg/L). Asterisks (*) indicate a significant difference between Accell Clean and PES-51 LC_{50} values and crosses ($^{+}$) indicate a significant difference between adult and larval shrimp LC_{50} values (Wheeler ratio test $p<0.05$).

Life Stage	**LC_{50} mg/L (95% CI)**	
	Accell Clean	**PES-51**
Adult	44.18 (30.39-60.52)	38.75 (17.99-65.43)
Larvae	48.64 (41.62-80.62)	155.42 (127.43-200.28)*$^{+}$

Table 2: Measured TEH and total PAH concentrations for the Accell Clean CEWAF and PES-51 CEWAF. <DL indicates values were less than the analytical detection limit.

Accell Clean (% CEWAF)	**TEH (mg/L)**	**Total PAH (µg/L)**
0	<DL	<DL
0.2	<DL	2.56
0.6	0.34	7.14
1.9	0.36	18.11
5.6	0.81	56.19
16.7	1.53	100.66
50	16.51	412.21
100	72.34	951.08
PES-51 (% CEWAF)	**TEH (mg/L)**	**Total PAH (µg/L)**
0	<DL	<DL
0.6	<DL	5.17
1.9	<DL	10.79
5.6	<DL	14.78
16.7	0.57	37.14
50	3.24	93.30
100	7.60	528.50

Table 3: Summary of 96-h LC_{50} values (and corresponding 95% confidence intervals) for adult and larval grass shrimp, *Palaemonetes pugio*, using measured TEH concentrations (mg/L) and measured total PAH concentrations (µg/L) in the CEWAF treatments. Asterisks (*) indicate a significant difference between Accell Clean and PES-51 CEWAF LC_{50} values and the crosses ($^{+}$) indicate a significant difference between adult and larval shrimp LC_{50} values (Wheeler ratio test $p<0.05$).

	LC_{50} (mg/L TEH) (95% CI)	
Life Stage	**Accell Clean CEWAF**	**PES-51 CEWAF**
Adult	1.86 (1.51-3.86)*	>7.6
Larvae	1.14 (1.01-1.28)*$^{+}$	>7.6
	LC_{50} (µg/L PAH) (95% CI)	
Life Stage	**Accell Clean CEWAF**	**PES-51 CEWAF**
Adult	113.99 (98.98-247.97)*	>528.50
Larvae	80.61 (33.13-106.76)*$^{+}$	>528.50

Table 4: Glutathione levels for adult grass shrimp after 96-h exposure to Accell Clean SWA and PES-51, as individual products and as CEWAFs. Asterisks (*) indicate significant difference from the control. The number of individual shrimp analyzed per treatment is shown in brackets.

Shoreline Cleaner Treatment (mg/L)	**Mean glutathione (nmol/g wet weight) mean (± SE) [n]**	**CEWAF Treatment (%)**	**Mean glutathione (nmol/g wet weight) mean (± SE) [n]**
Accell Clean SWA		Accell Clean SWA - CEWAF	
0	257.34 (29.09) [6]	0	183.53 (16.42) [6]
4.1	292.32 (27.60) [6]	0.2	223.24 (23.08) [6]
12.3	313.94 (30.14) [6]	0.6	228.41 (26.42) [6]
37	632.68 (76.86)* [3]	1.9	221.09 (14.05) [6]
111	602.44 (257.56)* [2]	5.6	229.42 (23.76) [6]
		16.7	339.29 (35.88)* [6]
PES-51		PES-51 -CEWAF	
0	333.12 (37.79) [6]	0	196.20 (28.95) [6]
4.1	357.00 (26.41) [6]	0.6	189.30 (14.62) [6]
12.3	337.78 (30.23) [6]	1.9	208.83 (29.03) [6]
37	283.38 (82.97) [3]	5.6	237.85 (10.07) [6]
111	322.44 (0.00) [1]	16.7	232.93 (15.15) [6]
		50	218.20 (31.00) [6]
		100	345.03 (18.55)* [6]

Table 5: Ecdysteroid activity for larval grass shrimp after 96-h exposure to Accell Clean SWA and PES-51, as individual products and as CEWAFs. Asterisk (*) indicates significant difference from the control. Cross (+) indicates LOEC for signficant monotonic trend (Williams test). The sample size (n) per treatment (7-10 shrimp were pooled to form a sample) is shown in brackets.

Shoreline Cleaner Treatment (mg/L)	**Mean ecdysteroid (ng 20-HE/g wet weight x 10^3) (± SE)**	**CEWAF Treatment (%)**	**Mean ecdysteroid (ng 20-HE/g wet weight x 10^3) (± SE)**
Accell Clean SWA		Accell Clean SWA - CEWAF	
0	56.0 (7.00) [3]	0	149 (19.2) [3]
4.1	80.0 (14.0) [3]	0.2	99.8 (15.0) [3]
12.3	62.2 (10.4) [3]	0.6	98.9 (8.59) [3]
37	134 (18.3)* [3]	1.9	179 (30.7) [3]
		5.6	203 (19.6) $^+$[3]
PES-51		PES-51 -CEWAF	
0	164 (18.9) [3]	0	174 (83.6) [3]
12.3	162 (43.6) [3]	0.6	23.8 (6.94) [3]
37	188 (18.9) [3]	1.9	73.7 (29.6) [3]
111	95.2 (9.57) [2]	5.6	39.1 (7.58) [3]
		16.7	59.6 (14.1) [3]
		50	86.5 (12.0) [3]
		100	69.4 (16.9) [2]

Table 6: Grass shrimp development at the end of the larval stage after 96-h exposure to Accell Clean and PES-51. Asterisks (*) indicate significant difference from the control. The number of individual shrimp analyzed per treatment (n) is shown in brackets.

Accell Clean (mg/L)	**n**	**Dry Weight (µg) mean (± SE)**	**Days to Postlarvae mean (± SE)**	**Number of Molts mean (± SE)**
0	[15]	707.7 (23.0)	16.4 (0.5)	7.1 (0.3)
4.1	[10]	776.6 (35.4)	15.4 (0.4)	6.4 (0.2)
12.3	[10]	807.4 (38.5)	14.9 (0.3)*	5.8 (0.3)*
37	[18]	751.2 (21.6)	16.2 (0.3)	6.5 (0.3)
PES-51 (mg/L)	**n**	**Dry Weight (µg) mean (± SE)**	**Days to Postlarvae mean (± SE)**	**Number of Molts mean (± SE)**
0	[14]	893.1 (27.9)	18.8 (0.6)	8.4 (0.3)
12.3	[9]	820.0 (34.6)	18.8 (0.4)	8.0 (0.3)
37	[14]	811.8 (30.8)	19.9 (0.6)	8.5 (0.2)
111	[3]	836.7 (31.9)	21.7 (0.3)	9.0 (0.0)
333	[1]	920.0 (0.0)	22.0 (0.0)	ND

Table 7: Grass shrimp development at the end of the larval stage after 96-h exposure to Accell Clean and PES-51 as CEWAFS. Asterisks (*) indicate significant difference from the control. The number of individual shrimp analyzed per treatment (n) is shown in brackets.

Accell Clean - CEWAF (%CEWAF)	**n**	**Dry Weight (µg) mean (± SE)**	**Days to Postlarvae mean (± SE)**	**Number of Molts mean (± SE)**
0	[17]	745.4 (22.8)	16.6 (0.4)	6.4 (0.3)
0.2	[18]	800.0 (28.7)	17.6 (0.3)	6.8 (0.2)
0.6	[18]	718.3 (28.1)	16.5 (0.4)	6.6 (0.2)
1.9	[17]	735.0 (26.2)	16.7 (0.23	6.9 (0.2)
5.6	[15]	858.2 (29.3)*	18.9 (0.3)*	7.7 (0.2)*
PES-51 - CEWAF (%CEWAF)	**n**	**Dry Weight (µg) mean (± SE)**	**Days to Postlarvae mean (± SE)**	**Number of Molts mean (± SE)**
0	[18]	730.2 (21.9)	22.6 (0.5)	5.9 (0.2)
0.6	[17]	730.2 (22.9)	24.1 (0.7)	6.2 (0.2)
1.9	[17]	745.7 (18.8)	22.4 (0.4)	5.6 (0.2)
5.6	[17]	727.4 (15.3)	23.5 (0.4)	5.9 (0.2)
16.7	[15]	761.2 (17.9)	23.9 (0.5)	5.9 (0.3)
50	[18]	728.9 (16.8)	24.1 (0.3)	6.3(0.1)
100	[6]	784.5 (33.5)	27.8 (1.0)*	7.2 (0.3)*

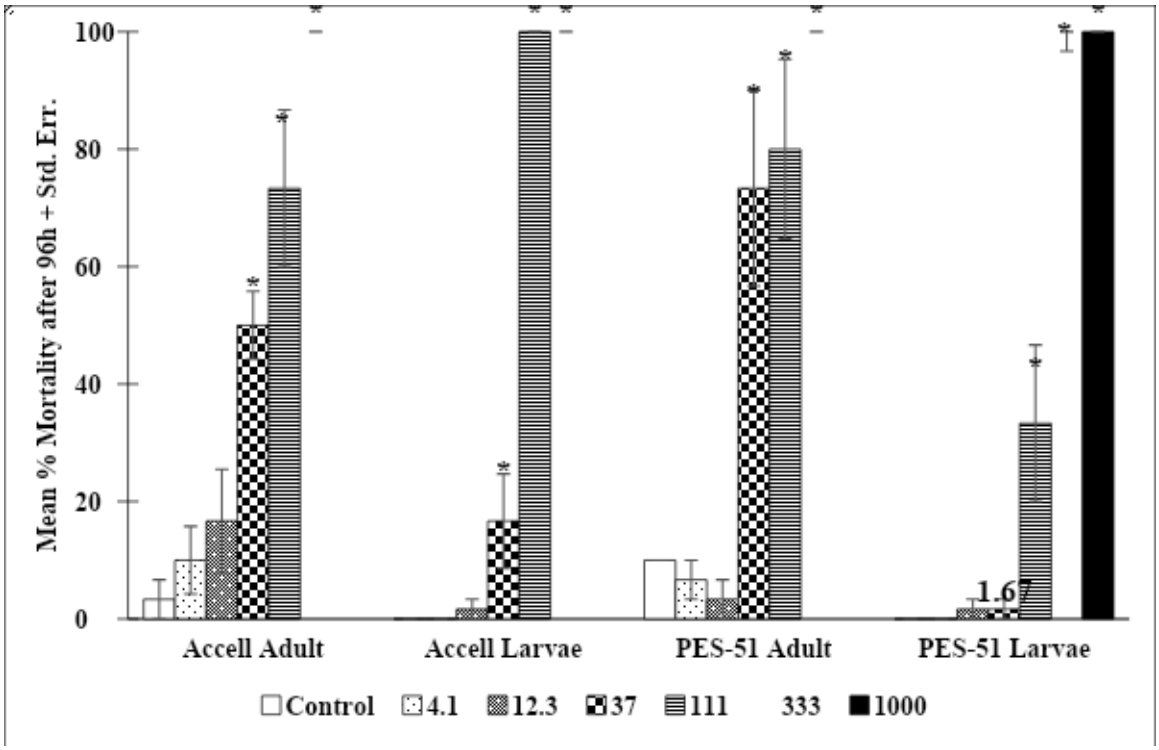

Figure 1: Adult and larval grass shrimp mortality after 96-h laboratory exposure to shoreline cleaners (mg/L) only. Only larval shrimp exposed to PES-51 were exposed at 1000 mg/L and these shrimp were not exposed at 4.1 mg/L. There were three replicates per treatment of ten shrimp each. Asterisks (*) indicate significant differences from the control, Dunnett's test. ANOVA p values for each experiment were <0.0001.

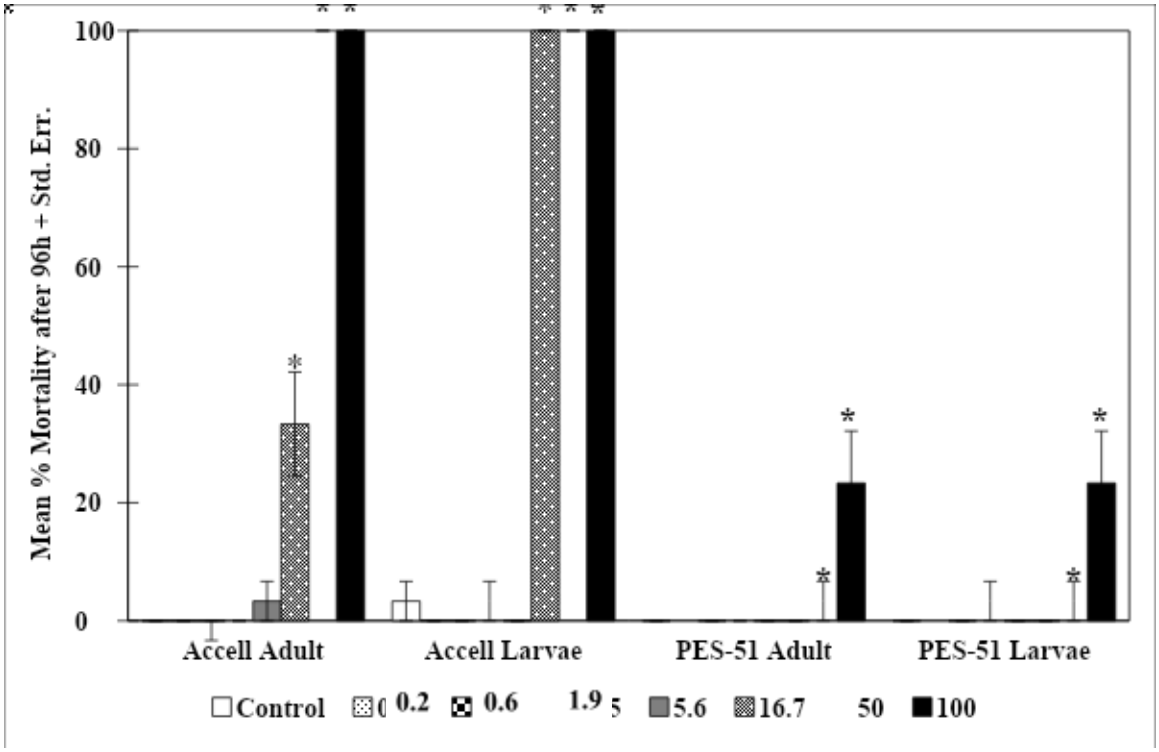

Figure 2: Adult and larval grass shrimp mortality after 96-h laboratory exposure to shoreline cleaner-CEWAF (% CEWAF). There were three replicates per treatment of ten shrimp each. Asterisks (*) indicate significant differences from the control, Dunnett's test (ANOVA: Accell adult $p<0.0001$; Accell larvae $p<0.0001$; PES-51 adult $p=0.0029$; PES-51 larvae $p=0.0056$).

Appendix 1. List of individual and alkylated PAHs that are included in Total PAH reported.

Individual and Alkylated PAHs in Total PAH	
naphthalene	C1-Naphthalenes
biphenyl	C2-Naphthalenes
acenaphthene	C3-Naphthalenes
acenaphthylene	C4-Naphthalenes
fluorene	C1-Fluorenes
dibenzofuran	C2-Fluorenes
dibenzothiophene	C3-Fluorenes
phenanthrene	C1-Dibenzothiophenes
anthracene	C2-Dibenzothiophenes
fluoranthene	C3-Dibenzothiophenes
pyrene	C4-Dibenzothiophenes
benz(a)anthracene	C1-Phenanthrenes/Anthracenes
benzo(b)naphtho(2,1-d)thiophene	C2-Phenanthrenes/Anthracenes
chrysene + triphenylene	C3-Phenanthrenes/Anthracenes
benzo(a)fluoranthene	C4-Phenanthrenes/Anthracenes
benzo(b)fluoranthene	C1-Fluoranthenes/Pyrenes
benzo(j)fluoranthene	C2-Fluoranthenes/Pyrenes
benzo(k)fluoranthene	C3-Fluoranthenes/Pyrenes
benzo(a)pyrene	C4-Fluoranthenes/Pyrenes
benzo(e)pyrene	C1-Chrysene/Benzanthracene
dibenzo(a,h)anthracene	C2-Chrysene/Benzanthracene
indeno(1,2,3-c,d)pyrene	C3-Chrysene/Benzanthracene
benzo(g,h,i)perylene	C4-Chrysene/Benzanthracene
	C1-Naphthobenzothiophenes
	C2-Naphthobenzothiophenes
	C3-Naphthobenzothiophenes
	C4-Naphthobenzothiophenes