Utility of Meiobenthos for Risk Assessment of Low-Level Crude Oil WAFs: Rapid Copepod-Based Approaches for Evaluating Reproductive and Population-Level Toxicity

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by

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Abstract

Crude oil contamination in coastal ecosystems serves as a source of water-accommodated hydrocarbon fraction (WAF) to porewater, posing acute and chronic risks to benthic invertebrates. Acute risk assessment methods are well developed for crude/fuel oils. Chronic risk assessment is poorly developed. This project developed for managers a new NIST-referenced full-lifecycle chronic bioassay for benthic copepods that yields data well suited for population growth/dynamics modeling and prediction. NIST standard and South Louisiana Sweet crude-oil were used to assess the effects of low level crude oil- WAF on the copepod *Amphiascus tenuiremis* survival, development and reproduction. Effects were assessed using a 96-well microplate full life-cycle test. This test allows tracking of individuals from the nauplius to the stage-I juvenile copepodite stage to sexual maturation and reproduction through female extrusion of the second brood. Briefly, 24-hour hatched nauplii were followed to adulthood (n_i= ≥120 nauplii/treatment) in individual glass-coated microplate wells containing 200 µL of solution. Treatments consisted of 10%, 30%, 50% and 100%-WAF, and seawater used as a control. Nauplii were monitored through development to adulthood, and sexually mature virgin copepods were mated in wells containing original treatments. Toxicological endpoints included mortality, development, sex ratios, fertilization success, viable offspring production per female, egg quality and population growth trajectories. An additional test using control and 10%, 30% and 50%-NIST WAFs (n_i= ≥60 nauplius/treatment) was conducted to assess the effects of UV light on nauplius survival and development. In exposures to 100% NIST and Louisiana-WAF, nauplius survival was significantly reduced (73± 6% and 32± 5%, respectively; $p<0.05$), relative to controls (92± 1% and 88± 3%, respectively); while copepodite survival was only reduced in the 50% Louisiana-WAF (88± 3%; $p=0.04$) relative to controls (98± 3%). Analysis of developmental curves showed that nauplii in the 10% NIST and Louisiana-WAF developed into copepodites at a faster rate, while nauplii in the 50% NIST and Louisiana-WAF, and 100%-NIST WAF developed at a slower rate than controls. Although the developmental rate in 100% Louisiana-WAF was similar to that of controls, nauplii showed a consistent 1.4 day delay in development into copepodites. Developmental delays/enhancements in the copepodite-to-adult window were also observed in exposures to both crude oil WAFs. None of the NIST WAFs had any significant effects on either reproductive success or total viable production ($p>0.05$). In contrast, reproductive failure was increased by 30% and 41% in 30% and 100% Louisiana-WAF, respectively, compared to controls (3.33± 4.7%). However, none of the Louisiana WAFs had any effects on either embryo hatching success or total viable production ($p>0.05$). Leslie matrix projections showed lower naupliar abundance in exposures to 100%- NIST WAF and to all Louisiana WAFs, compared to controls. The results from the UV/Non-UV test showed that 30% and 50% NIST-WAFs in combination with UV were sufficient to cause negative impacts on naupliar survival and development. Chemical analysis of freshly extracted NIST and Louisiana WAFs indicated that naphthalene was the most abundant PAH (>50%) in both WAFs. Nauplius survival and copepod developmental endpoints were the most sensitive indicators of exposure to crude-oil WAFs. These results demonstrate the utility of a logistically simple, rapid (25 days) copepod full-lifecycle (egg-to-egg) bioassay for evaluating exposures to low-level WAF concentrations typical of *post-remediation* sediment or beach porewaters. The testing methodology presented here can be easily transferred/taught to other scientists, technical staff

and environmental managers as a quick and accurate way to predict chronic low-level (threshold) toxicity of crude oil WAFs.

Key words: Copepod bioassay; Crude oil WAF; PAH toxicity; UV toxicity; *Amphiascus*.

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1.0 Introduction

Oil spills on beaches, mudflats, and in salt marshes threaten flora and fauna via mechanical fouling, contact toxicity, and especially via the water- accommodated hydrocarbon fraction (WAF) in surface and porewaters (NRC 1985). This WAF is acutely to chronically toxic to lower trophic level organisms (e.g., phytoplankters and copepods in marine systems), and may be bioaccumulated to lipids (Klosterhaus et al. 2002) and potentially transferred up to higher predators. The meiobenthic community, comprised of nematodes, harpacticoid copepods and foraminifera (Coull and Chandler 2001), is usually the first to show impacts from crude oil spills (Coull and Chandler 1992). Of the multiple meiofaunal taxonomic groups, harpacticoid copepods are the most sensitive meiobenthic taxon to crude oil in water and sediment (Table 1). Studies to date showing elevated copepod sensitivities to oil have ranged from simple *in vitro* LC_{50}-type tests of straight crude and fuel oils, to more elaborate micro/mesocosm (e.g., Carman et al. 1995) and manipulative field studies (e.g., Fleeger and Chandler 1983). The most toxic fraction of crude oil and crude oil-WAF to benthos is the low molecular weight and polycyclic aromatic hydrocarbon (PAH) fractions (NRC 1985). In the field, low molecular weight compounds (e.g., naphthalenes, short-chained aliphatics, benzene) in crude oil usually evaporate within days of introduction, and it may be argued that these compounds have minimal lasting impact on sediment-based communities. For a rapidly growing/reproducing meiobenthic copepod population, however, such "short" exposures are actually a significant proportion of copepod lifecycle and are sufficiently long to negatively impact survival and especially reproduction. For example, a suspended layer of crude oil caused 100% mortality of the tide pool harpacticoid *Tigriopus californicus* in 3-7 days (Kontogiannis and Barnett 1973). For a WAF, only 200 ppb caused 50% mortality of *Halectinosoma curticorne* in six days (Gyllenberg 1986); more importantly, the same concentration reduced brood size of *Nitocra affinis* by 43% (Ustach 1979). For a highly refined gasoline fuel oil, Tarkpea and Svanberg (1982) found that the WAF with and without low molecular weight fuel additives was an order of magnitude less toxic to *Nitocra spinipes* (96-h LC_{50}= 200 mg/L) than raw crude oil was to other species (Kontogiannis and Barnett 1973; Gyllenberg 1986). In field mesocosms, Boucher et al. (1981) mixed crude oil directly with sediments at 10- and 100-g oil/Kg-sediment and monitored subsequent changes in meiofaunal densities for 180 days. Copepods decreased significantly (>50%) by 20 days exposure in the 100 g/Kg treatments and did not recover.

Why Meiobenthic Copepods as a Tool for Crude Oil Risk Assessment? Based on the published data, harpacticoid copepods may be the meiofaunal taxon of choice for oil impact/effects studies because of their greater toxicant sensitivity, ease of taxonomic identification, ease in experimental manipulations (e.g., Carman et al. 1995), and their culturability in the laboratory. In addition, they are extremely important as potential trophic vectors of hydrocarbon bioaccumulation (Wirth et al. 1994; Klosterhaus et al. 2002, 2003) and movement into benthic-based food-webs (Gee 1989, Coull 1990). Harpacticoid copepods are lipid rich (5-12% by weight), and body burdens of lipophilic PAHs can easily reach ug/g concentrations in PAH-exposed field populations (Klosterhaus et al. 2002). Benthic copepods are typically the second most abundant metazoan invertebrate in sediments world-wide (Hicks and Coull 1983). The entire lifecycle of most temperate to tropical copepods takes place in 15-25 days (species dependent) which makes them similar to freshwater daphniids in their utility for *marine*

ecotoxicology. Meiobenthic copepods are indigenous to every oxic sedimentary habitat studied on the planet to date (Hicks & Coull 1983), and they are also intimately associated with all compartments of the sedimentary matrix throughout their lifecycles (i.e., they have no pelagic lifecycle stage). As such, they are particularly vulnerable to particulate- and porewater- borne toxicity (Green et al. 1993; Hagopian et al. 2001), and can be useful sentinels of oil pollution that penetrates into the interstices of sandy beaches (Hennig et al. 1983).

2.0 Objectives

Acute toxicity test methods are well developed for water-borne toxicants generally. For chronic crude oil exposures however, few monitoring/testing tools have been available to robustly determine the threshold WAF concentrations below which bioaccumulation and toxicity are not a chronic management concern in coastal ecosystems. This is especially true for population-level impacts because most laboratory and field bioassay organisms in current use have long, logistically untenable lifecycles. One frequently overlooked, but ecologically important, lower trophic-level community of high potential value in this regard is the rapidly reproducing meiobenthic copepods of nearshore systems. **This research project developed for managers a new full-lifecycle testing technology for individual to population-level determinations of "safe" WAF crude oil concentrations using meiobenthic copepods.** In the current study, we employed the rapidly maturing harpacticoid copepod *Amphiascus tenuiremis* as a model for assessing the life-history effects from exposure to petroleum crude oil-WAFs, with exposures designed to simulate post-remediation WAF conditions. This model copepod allows the evaluation of WAF impacts on several lifecycle endpoints (i.e., development and reproduction), as well as the assessment of potential population-level effects under controlled laboratory conditions. The well accepted Leslie matrix modeling approach to population growth prediction was employed to temporally project WAF impacts on population size over multi-generational exposure times. **The chronic toxicity test protocols developed here provide rapid and sensitive measurements of crude oil-WAF effects on estuarine meiobenthic copepods specifically. However, as copepods are crustaceans with similar ecophysiologies as their macrofaunal cousins – shrimps and crabs – this new approach is useful for protecting more commercially important species as well.**

3.0 Materials and Methods

Test Organism

Amphiascus tenuiremis is an infaunal sediment-dwelling harpacticoid copepod that inhabits muddy inter- and subtidal estuarine sediments from the Baltic Sea to the southern Gulf of Mexico (Lang 1948). *A. tenuiremis* has a generation time of 21 days (egg-to-egg) at 20°C in sediment (Chandler and Green 1996), and a lifecycle consisting of nauplius (6 stages) and copepodites (5 stages), and sexually dimorphic adults.

Preparation of Crude Oil-WAF

South Louisiana Sweet and "NIST standard" petroleum crude oil were used to assess the effects of WAF on copepod lifecycle test endpoints. Standard petroleum crude oil was purchased from the National Institute of Standards and Technology (SRM-1582; NIST; Washington, DC). Briefly, 0.22 µm- filtered seawater (30‰; 180 mL) and a Teflon-coated stirring bar (1 cm) were placed in each of three amber round glass bottles (250 mL), following the transfer of Louisiana or NIST crude oil (6 mL) dropwise to the top of the seawater surface. Bottles were sealed tight with Teflon-lined screw open-top caps, and headspace air manually purged through screw-top septa with a stainless-steel needle attached to a 50 mL Hamilton gastight syringe (Fisher-Pittsburgh, PA). The headspace was flooded and re-filled with nitrogen (>99% purity) to prevent degradation of oil and bottles sealed with Teflon tape. Bottles were placed in an incubator on a magnetic stirrer plate and were stirred in the dark for 36h at 20 ºC. WAF was collected by syringe after 36-hours and transferred to clean amber bottles. Air was purged as described before and nitrogen was added to the headspace prior to storage. Amber bottles containing extracted WAF were covered with aluminum foil and stored in darkness at 4 °C. WAF samples for chronic exposures were withdrawn from bottles using a gastight syringe and water volume replaced with nitrogen to minimize loss/degradation of WAF. WAF stocks were chemically stable throughout the duration of the experiment (~25d).

Dilution series exposures

WAF treatments included 10%, 30%, 50%, and 100%-WAF treatments, with filtered (0.22 µm) and fully aerated seawater (30‰) used as a control (0%-WAF). Sixty mL of 100% full strength WAF was withdrawn from WAF amber bottle stocks and poured into methylene chloride (>99.5 dichloromethane, HPLC grade; Fisher) rinsed 250 mL-beakers. Dilution treatments were made in 100 mL beakers by combining appropriate amounts of seawater and 100%- WAF. Wells were loaded with 200 µL of WAFs or control solution and the microplates placed in an incubator under fluorescent light.

Crude Oil-WAF chronic exposures

Copepod full-lifecycle microplate bioassays (ASTM E 2317-04) were conducted to assess the effects of low-level NIST and South Louisiana Sweet crude oil- WAF on *A. tenuiremis* development and reproduction (Fig. 1). Gravid *A. tenuiremis* were collected from monoculture sediments in the laboratory and transferred to a 12-well plate containing seawater and 75 µm mesh cup inserts. The inserts retain the females while allowing hatching nauplii to fall to the well bottom over a 24-h period.

Nauplii were gently placed individually into 250 µL microplate wells (i.e., glass coated 96-well microplates; Sun-SRI; Duluth, GA). Each microplate was loaded with ≥ 40 nauplii and haphazardly assigned in triplicate to each of the treatments or controls. Excess transfer water was removed from each well previous to the addition of 200 µL control or WAF treatment solutions. Nauplii were monitored daily through stage-I copepodite stage to sexual maturity. Upon reaching sexual differentiation, virgin male and female copepods were removed from wells and mated pair-wise in new wells containing original treatments. Treatment solutions were replaced (>90% water replacement) every third day throughout the experiment with fresh treatment solutions

(>90% dissolved oxygen (DO)) to ensure proper water quality and consistent WAF or control exposure. Water quality (salinity, temperature, DO and pH) in fresh test solutions and controls were recorded prior to each water change. Individuals throughout the duration of the experiment were fed every 6d with 3 µL of a fresh algae mixture (10^7 cells/mL of 1:1 of *Isochrysis galbana*:*Dunaliella tertiolecta*). Covered microplates were held in an incubator (Revco; Asheville, NC) at 25±1 °C and 12h:12h light:dark conditions.

Survival and developmental times were recorded daily for individual naupliar and subsequent copepodite stages via inverted stereomicroscopes. Developmental endpoints included naupliar survival and development-time to the stage-I copepodite stage, copepodite survival and developmental time to successful sexual differentiation, and sex ratios. Likewise, each mating pair was monitored during the mating period which was allowed to last up to 8d post mating to accommodate potential delays in reproduction. Reproductive endpoints included reproductive success/failure, first and second brood sizes, hatching success and total viable offspring production. Reproductive success was defined as those mating pairs unable to extrude viable embryos over the entire mating period (8d).

Stage-Structured Population Growth Model

Multi-generational population-level effects of crude oil-WAF were estimated using empirical microplate data fitted to a matriarchal stage-structured Leslie matrix model ((RAMAS® EcoLab 2.0; Applied Biomathematics; Setauket, NY) Akçakaya et al 1999; Caswell, 2001). Naupliar production projections were based on: (1) stage-specific survival rates, (2) the proportion of copepodites developing into virgin females, (3) the proportion of females able to reproduce, and (4) female fecundity (i.e., viable hatched offspring/female) through two broods. Model constraints included demographic stochasticity and an arbitrary carrying capacity of 20,000 individuals. Empirical data from each microplate per treatment/control were modeled (through 10 model runs) to project naupliar production through 3 generations. Since simulations were run per each replicate microplate and averaged within treatments, subsequent statistical comparisons of population-growth predictions across WAF treatments and controls could be accomplished.

Ultraviolet and Fluorescent Light Exposures.

A partial-lifecycle (i.e., naupliar-to-copepodite stage only) microplate bioassay was also performed to assess the potential UV effects in combination with NIST-WAF exposures. A new batch of NIST crude oil-WAF was prepared as described above with exposure media including 50%, 30%, 10% strength-WAF and seawater controls (0%- WAF). Experimental setup was performed as described above with three plates per treatment loaded with > 20 hatched nauplii per microplate. Exposures were done under dual fluorescent and ultraviolet (UV) light conditions. Wells were loaded with 200 µL of WAF or control solution and microplates haphazardly assigned in triplicate to either fluorescent or UV exposure regimes. The incubator was designed to accommodate dual sets of lamps. UV and fluorescent lamps were placed above and below the exposure worktable, respectively. Microplates assigned to the fluorescent exposure regime were protected from UV light by covering the microplate with aluminum foil. Lids from the microplates assigned to the UV regime were removed and replaced with 6x5 cm glass lantern slides to allow for infiltration of UV light. Both fluorescent and UV light was measured using an actinometer, with UV-A and UV-B measured between 290-320 nm and 320-

400 nm, respectively. Total UV radiation (UV-A plus UV-B) was adjusted to simulate 25% of the total natural sunlight UV (1500 $\mu W/cm^2$) of a summer solstice at Murrells Inlet, SC. Endpoints in this partial-life cycle bioassay included naupliar lethality over time, and naupliar development to stage-I copepodite.

Statistical Analyses

Naupliar-to-copepodite and copepodite-to-adult developmental curves between WAFs and controls were compared using Generalized Linear Interactive Modeling (GLiM) (Piegorsch and Bailer 1997) fit via PROC GENMOD (SAS® Institute, Inc.; Cary, NC). Linear models do not adequately describe variable relationships when: 1) the distribution of the response variable does not have a continuous (i.e., normal) distribution; and/or 2) the effects of the predictors on the dependent variable are not linear in nature (Piegorsch and Bailer 1997). In these cases, variable relationships are better explained with GLiM models. GLiM uses generalizations of normality-based linear models (i.e., ANOVA and linear regression) to account for non-normal responses (Piegorsch and Bailer 1997). In this study, a model that describes developmental curves (i.e., Probit link; Piegorsch and Bailer 1997, Chapter 7) was used to generate regression estimators (i.e., slope and intercept), and then comparisons were made between slopes and intercepts of individual WAF treatments and the control curves based on the logistic regression (LR) deviation statistic (LR; Piegorsch and Bailer 1997, Chapter 7). In this way, the entire full-lifecycle response curves could be compared statistically to the control response, rather than comparing single arbitrary response points along pairwise temporally-defined curve(s).

All lifecycle bioassay endpoints were tested for normality and homogeneity, and variables failing normality were transformed accordingly. For the full-lifecycle microplate bioassay, differences in stage-specific survival, percent mating success, first and second brood sizes, hatching success and total viable offspring production between WAF-exposed individuals and controls were performed by a one-way analysis of variance (PROC GLM, SAS) using the Bonferroni adjustment test for multiple pair-wise comparisons (SAS 2004).

Analysis of the WAF-UV vs. non-UV partial lifecycle data included naupliar mortality curves over time where Probit Analysis (PROC PROBIT, SAS) was employed to estimate median time to lethality (LT_{50}) values (Piegorsch and Bailer 1997). The log likelihood ratio Chi-square test statistic (LR) was used to evaluate the goodness of fit between naupliar mortality curves and the predicted Probit model. Also, naupliar-to-copepodite developmental curve analysis within WAFs and across light regimes was performed using GLiM, as described above.

Population-level effects of WAF (full-lifecycle bioassay only) were analyzed using population projections from individual microplates per WAF treatment and control, with variance estimates computed at the level of individual microplates. Data were logarithmically transformed (i.e., $Log_{10}(x+1)$), and differences in projections across WAF and controls were determined by a one-way analysis of variance (PROC GLM, SAS). All tests for significance were performed using an alpha (p) level ≤ 0.05.

Water Chemistry Analysis

Three freshly made 100%-WAF stocks (100 mL/each) were analyzed for PAHs following EPA SW-846 Method 3510C. Briefly, 100 mL WAF stock and seawater control samples were transferred to 250 mL separation funnels, followed by the addition of 50 µL of surrogate standard solution (2-fluorobiphenyl (96%) and p-terphenyl-d14 (98%), @ 200 µg/mL; Aldrich®, St. Louis, MO). PAHs from all solutions were extracted three times with methylene chloride (6 mL) by shaking the funnel vigorously for 2 min, and collecting the organic phase into a 40-mL borosilicate glass vial. The three combined extracts were filtered through a 60° Pyrex glass funnel equipped with Fisher 11.0 cm filter paper loaded with 2 g of anhydrous sodium sulfate. Samples were collected into 40 mL vials, blown down to 1 mL with a gentle stream of nitrogen, and the remaining sample transferred to a 2 mL volumetric flask. Following the addition of 50 µL internal standard (i.e., phenathrene-d10) and careful mixing of the volumetric flask contents, samples were transferred to 2 mL Target I-D™ vials and blown down with nitrogen to exactly 1 mL.

The extracts containing PAHs were analyzed using a Varian 3800 gas chromatograph (GC) coupled to a Varian Saturn 2000 MS/MS ion trap mass selective detector system (ITMS). Injection port and transfer line temperatures were set at 280°C. The GC column oven was programmed to 50°C (2 min hold) and ramped to 290 °C at 12 °C/min (10 min hold). The ion trap and the manifold temperature were set at 220° C and 80° C, respectively. A fused silica capillary column (30m) x 0.25 mm x 0.25 µm film thickness) (J&W Scientific, Folsom, CA) was used to separate target PAH analytes. Sixteen PAH analytes (2-6 ring structures) were quantified based on six-point calibration curves of a standard mixture (0.5 to 20 gm/mL; Supelco, St. Louis, MO) and two surrogate standards. A continuous calibration check standard and an instrument blank sample were analyzed before and after sample analyses. Method accuracy (i.e., percent recovery) and precision (i.e., sample percent difference) were monitored by analyzing multiple standard-mixture laboratory control samples. Instrument performance was verified by running a standard calibration check at the end of all sample analyses.

4.0 Results

Dilution Series Exposures

Survival. The full-lifecycle exposure (i.e., 24 h hatched nauplius to extrusion of two broods) of the copepod *A. tenuiremis* to NIST crude oil lasted a total of 30 d. One of the control microplates from this bioassay was removed from the analysis due to a plate manufacturing imperfection (i.e., it slowly leaked.). Control naupliar and copepodite survival was >90%; only the highest WAF strength, 100%-WAF, showed significantly elevated naupliar mortality (27±6%) compared to controls (0%-WAF) ($p = 0.01$). Both naupliar and copepodite survival in the 10%, 30% and 50%-WAF averaged > 87% and were not significantly different from control survival. No LC_{50} or LT_{50} estimates could be derived for NIST WAFs because of their mild acute toxicities.

The full-lifecycle exposure to the South Louisiana Sweet crude oil WAF lasted 32 d. Naupliar survival to the 1st copepodite stage across most WAF treatments was >75% and not significantly

different from controls (88± 3%; p >0.05; Fig. 2); however, naupliar survival in the 100%-WAF was reduced by 57% relative to controls (p <0.001). The only treatment showing acute, time dependent naupliar toxicity was the 100%-WAF where the predicted median time to lethality (LT_{50}) was 4 d. Copepodite survival to adult, on the other hand, was > 90% in most WAF treatments and only significantly reduced in the 50%-WAF (88± 3%; p =0.04) compared to controls (98± 3%).

Development. GLiM analysis of naupliar-to-copepodite developmental curves in NIST WAF exposures predicted 10-13 d for 80% naupliar development into copepodites across WAF dilutions and the controls. Curve analysis, based on slopes and intercepts, indicated that most WAF dilutions, except for 30%-WAF, showed developmental curves different from the controls. Nauplii exposed to 10%-WAF showed a much faster development, while nauplii in the 50% and 100%-WAF showed a much slower development into the copepodite stage (Fig.3A). Also, in the copepodite-to-adult developmental curves, those copepodites exposed to 100%-WAF showed a 2 d and 4 d delay in development into adult female and male copepods, respectively, compared to control copepodites (Fig. 3B). Only copepodites exposed to 30% and 100%-WAF showed much slower development into adult males than controls.

GLiM analysis of the developmental curves from naupliar-to-1st copepodite stage in South Louisiana Sweet WAF exposures predicted 11.5 to 12 d for 80% development across WAF dilutions and the controls. Curve analysis, based on slopes and intercepts (Fig. 4A) indicated that nauplii in the 10% and 50%-WAFs showed developmental curves significantly different from controls. The majority of nauplii exposed to 10%-WAF developed into copepodites 46% faster on average, while in exposures to 50%-WAF they developed 15% slower than controls. Although the regression analysis of the 100%-WAF was not significantly different from controls, these nauplii showed a consistent 1.4 d delay in development into copepodites compared to controls. Similar patterns were also observed in the copepodite-to-adult development window (Fig. 4B). Copepodites exposed to 10% and 50%-WAF developed on average 1.8 d earlier and 2.2 d later into females, respectively, compared to controls (i.e., 80% development = 10.2 ± 0.5 d). In the same treatments, copepodites developed on average 1.2 and 1.9 d later into males, respectively, compared to controls (i.e., 80% development= 8.5± 0.3 d). Similarly, although regression analysis of copepodite development in the 100%-WAF was not significantly different from the controls, these copepodites showed a consistent 1.5 and 2.1d delay in development into females and males (data not shown), respectively, compared to controls.

Reproduction. Female:male ratios in NIST WAF exposures were variable across microplates and not significantly different between WAF-treatments and the controls. Female:male ratios ranged from 65%:35% to 28%:72%. The number of mating pairs per treatment unable to reproduce viable offspring during a 9 d mating period was highly variable across treatments. Reproductive failure, defined as the percent of females unable to produce at least two viable broods, ranged from a low of 14.7± 3.5% in controls to a high 33.7± 12.5% failure for the 100%-WAF (Table 2). However, reproductive failure was not significantly different between WAF treatments and the controls (p>0.05). Embryo hatching success over two broods was 87± 19.4% in controls. Only the 100%-WAF treatment significantly reduced hatching success (70.0± 21.2%; p=0.0035) compared to controls. Hatching success in the remaining WAF treatments was >89% and not significantly different from control hatching (p>0.05). Also, total viable offspring in control

females pooled over two consecutive broods was on average 12.9± 4.1 nauplius per female. None of the WAF treatments had statistically significant effects on viable offspring ($p>0.05$) compared to the controls. Total viable offspring in the 10%, 30%, 50% and 100%-WAF were 14.5± 4.3, 11.7± 4.7, 14.5± 4.7 and 15.4± 4.5 nauplii per female, respectively.

Considering the developmental times for each of the crucial life and reproductive stages (i.e., naupliar-to-stage I copepodite, stage I copepodite-to female, mating-to-2nd brood hatch), all WAF treatments, except for the 50%-WAF, showed a significantly different full-life developmental time period (i.e., nauplius to 2nd brood extrusion) ($p<0.05$) compared to controls (Table 2). Individuals exposed to 10%-WAF and 30%-WAF showed on average an overall full-life development time of 3 d ($p<0.0001$) and 1 d ($p=0.0086$) faster than controls, respectively; while individuals exposed to 100%-WAF showed on average a full-life development time 3.5 d later than controls ($p=0.001$).

Female-to-male ratios in South Louisiana Sweet WAF exposures were variable across microplates, ranging 47%:53% to 57%:43%, and were not significantly different between WAF treatments and controls ($p>0.05$). The number of mating pairs per treatment was variables across treatments, with the lowest number of pairs (7 pairs) in the 100%-WAF, resulting primarily from low nauplius to adult survival. Compared to controls, the percent females unable to produce at least two viable broods was significantly increased by 30% and 41% in exposures to 30% and 100%-WAF, respectively (Table 3). Despite this increase in reproductive failure, embryo hatching and total viable production of successful mating pairs was not significantly different among WAFs and controls ($p>0.05$).

Considering the developmental times for each of the crucial life and reproductive stages (i.e., naupliar-to- 1st copepodite stage, copepodite-to female, mating-to-2nd brood hatch), all WAF treatments, but the 30%-WAF, showed a significantly different full-life developmental time (i.e., nauplius to 2nd brood extrusion) ($p<0.05$) compared to controls (Table 3). Individuals exposed to 10%-WAF showed on average a full-life development time 1.8 d faster than controls; while individuals exposed to 50% and 100%-WAF showed on average a full-life development time 4 and 4.4 d later than controls, respectively.

Stage-Structured Population Growth Modeling. Predictions of potential population-level responses following exposures to crude oil WAF were obtained by applying a staged-based Leslie matrix model (Akçakaya et al 1999; Caswell 2001). Model projections were done using empirical data from each microplate per treatment, using estimated generation time (i.e., nauplius to 2nd brood extrusion; Tables 2 and 3) as timeframes for projected generations F_1, F_2 and F_3. Since sex ratios were variable and were not significantly altered by either NIST or South Louisiana Sweet WAF treatments (relative to controls), all population projections were performed using a 50% virgin male:female proportion for all WAF treatments and controls.

All naupliar projections through three generations were significantly different between NIST crude oil WAF treatments and the controls ($p<0.0001$). Across all three generations, the 10% -WAF, 30% -WAF and 50% -WAF showed higher naupliar production projections than controls (Fig. 5). Naupliar projections in the 10%, 30% and 50% -WAF were on average 16% higher than controls in the F_1, and between 41 and 48% higher than controls in the F_2 and F_3. In contrast,

naupliar projections in the 100% -WAF were on average 27%, 47% and 55% *lower* than controls in the F_1, F_2 and F_3, respectively. In addition, while the predicted time to reach the arbitrary carrying capacity of 20,000 individuals was 92.4 d for the control population, these times were *accelerated* by 18.3, 13.3 and 5 d in 10%, 30% and 50%-WAF, but delayed by 384 d in the 100%-WAF exposed population (Fig. 5).

Naupliar projections through three generations were consistently and significantly lower between South Louisiana Sweet crude oil WAF treatments and the controls (p<0.0001; Fig. 5). Compared to controls, naupliar production across all generations in the 10%, 30%, 50% and 100%-WAFs was 29±7, 34±7, 18±5 and 79±9% lower, respectively. In addition, while the estimated time to reach an arbitrarily chosen carrying capacity of 20,000 individuals was 75.4 d for the control population, these estimated times were *delayed* by 2.5, 8.5, 17 and 394 d in the 10%, 30%, 50% and 100%-WAF populations, respectively (Fig. 5).

UV and Fluorescent Light Exposures. Fluorescent light levels were 376±187 μW/cm^2, while total UV levels (UV-A and UV-B) were 355±24 μW/cm^2 with UV-B accounting for 13±0.7% of the total light irradiance. These UV levels were chosen to mimic coastal values at summer solstice in Murrells Inlet, SC.

Naupliar survival to stage-I copepodite throughout the 16 d partial-lifecycle bioassay was >80% under the fluorescent regime across NIST crude oil-WAFs and controls (p>0.05). In contrast, WAF-UV exposures resulted in reduced naupliar survival from a high 75.8± 11.2% in control microplates to a low 23.2± 9.2% in the highest WAF (p=0.031). Naupliar survival in the 10%- and 30%-WAF treatments under UV conditions was 56.8± 20.2% and 51.9± 10.5%, respectively, and not statistically significant from the controls (p>0.05). On average UV alone increased naupliar mortality by 15%, compared to mortality under fluorescent regimes only. Exposures to WAF, in combination with UV, resulted in a dose-dependent naupliar mortality not seen in the absence of UV (Fig. 6). Probit analysis fitted to raw naupliar mortality data (all p's > 0.05, LR Chi-Square Goodness-of-Fit test) indicated a median lethality time (LT_{50}) of 13.2 d (95% Confidence Interval = 12.1- 14.7 d) for the 30% and 9.3 d (95% CI= 8.5-10.1 d) for the 50%-WAF and UV interaction.

GLiM analysis of control naupliar-to-copepodite developmental curves predicted 12.5-14 d for 80% naupliar development into copepodites under both fluorescent and UV regimes. Within 30% and 50%-WAF treatments, analysis, based on slopes and intercepts, showed a significantly different developmental curve between the UV and fluorescent regimes (Fig 7). Nauplii exposed to 30% and 50%-WAF showed on average a 2 and 3 d delay in development, respectively, compared to nauplii under fluorescent conditions. Development times across all treatments under florescent conditions were similar to those from the dilution series exposure.

Comparison between NIST and South Louisiana Sweet WAF effects on A. tenuiremis. A summary of all the survival, developmental and reproductive endpoints where favorable or adverse effects on *A. tenuiremis* were observed following exposure to NIST and South Louisiana Sweet crude oil WAFs is presented in Table 4. The most consistent effects from exposures to NIST and Louisiana Sweet WAFs were *delayed* development in the highest WAF exposures, and *enhanced* nauplius-to-copepodite development rates in the lowest WAFs (especially for the least

toxic NIST standard). However, these and other negative effects were more pronounced in exposures to South Louisiana Sweet WAF which contained ~5-fold higher total PAHs.

Water chemistry analysis. NIST and South Louisiana Sweet 100%-WAFs were analyzed for a total of 16 PAH analytes (2-6 rings). Percent recovery of the surrogate standards, 2-fluorobiphenyl and p-terphenyl-d14, were consistent and acceptable across crude oil types: 90± 3% and 143± 4%, respectively, in NIST 100%-WAFs samples (n=3), and 79± 2% and 155± 6%, respectively, in South Louisiana Sweet samples (n=3). Relative percent differences (i.e., precision) across multiple samples were variable among PAHs and ranged from 1 to 18% . Of those 16 PAHs, 6 were detected in NIST-WAF, and 9 in the South Louisiana Sweet WAF. Naphthalene (2 rings), phenanthrene, fluoranthene and acenapthene comprised over 95% of the total PAHs in NIST and South Louisiana Sweet 100%-WAF, with the highest predominance (50%) being naphthalenes. The PAHs benzo-[b]-fluoranthene, benzo [k] fluoranthene, benzo [a] pyrene, benzo [g, h, i] perylene, dibenzo [a, h] anthracene, fluorene and ideno [1,2,3- c, d] pyrene were below instrument detection limits for individual PAHs or were non-detectable in NIST or South Louisiana Sweet WAFs. Seawater controls had non-detectable levels of PAHs. Although total PAH concentration in the South Louisiana Sweet WAF was six times greater (878± 28 µg/L) than that in NIST-WAF (134± 4 µg/L), the composition and proportion of analytes was quite similar between these two WAFs (Fig. 8). **If this compositional/proportional concordance holds true for NIST relative to other crude oil types [and it should given that it is a federally controlled standard], then the NIST Standard and this copepod bioassay could provide a useful "encyclopedia" of crude oil WAF individual- to population-level effects benchmarked against NIST for any crude oil of interest.**

5.0 Discussion

Coastal environments are the most vulnerable shoreline ecosystems to crude oil contamination (Gundlach and Hayes 1978). Oil spills near these areas threaten benthic fauna and flora as oil serves as a source of WAF to porewater. Among benthic fauna, meiobenthic crustaceans are particularly sensitive to oil contamination and the first community to show toxic effects (i.e., density decline of harpacticoid copepods) to oil exposure (Coull and Chandler 1992). Early studies have shown that even short exposures to crude oil and crude oil WAF could compromise copepod survival and population maintenance (Carman and Todaro 1996; Gyllenberg 1986).

In the current study, the 100% NIST and South Louisiana Sweet WAFs (total PAHs = 134.2± 4.4 µg/L and 878± 28 µg/L, respectively) not only increased naupliar mortality relative to controls, but also reduced significantly naupliar (plus the 50%-WAF) and copepodite development into the next life stage. In addition, delayed development particularly into males was also seen across most WAFs from both crude oils (i.e., 30%- and 100%- NIST, and 10%, 50%- and 100%- South Louisiana Sweet). Interestingly, development enhancement relative to controls was observed in the 'nauplius-to-copepodite' and 'copepodite-to female' developmental windows in 10%- NIST and South Louisiana Sweet WAFs, and 10%- South Louisiana Sweet WAF, respectively. Studies have indicated that high levels of contaminants such as hydrocarbons cause direct narcosis and can inhibit ecdysis in crustaceans (i.e., PAHs; Swartz et al. 1995; Kennish 1992), which in turn would result in mortality, and alteration of processes such as molting and development.

Naphthalenes, on the other hand, which were the major constituents in the WAFs (50%) and the most water soluble aromatic compounds in NIST WAF, are likely the most important contributors to the naupliar toxicity and developmental effects observed. Slower copepdodite development, particularly into males, is a result that is somewhat difficult to explain. A recent study with *A. tenuiremis* (Bejarano et al. *in press*) showed that males are generally more sensitive to acute and chronic organic contaminant toxicity than female copepods. In contrast, an enhancement in naupliar-to-copepodite development at 10%-WAF could potentially be explained by induction of cytochrome P450-dependent xenobiotic monooxygenase isozymes. Studies have suggested (Mothershead and Hale 1992; Oberdörster et al. 1999; Snyder 1998) that induction of P450-isozymes by relatively low levels of PAHs could result in an acceleration of the molt cycle (i.e., development) by increasing titers of the molting regulating hormone 20-hydroxyecdysone. For example, Snyder (1998) reported that the expression of hepatopancreas CYP45 during the molt cycle of the American lobster, *Homarus americanus*, mirrored hemolymph titers of ecdysteroids, indicating that this P450 is potentially involved in processes such as ecdysteroid metabolism, molting and development.

In this study, almost none of the NIST WAFs tested had any effects on viable offspring production. Embryo hatching success was the only reproductive endpoint reduced in the 100%-NIST WAF, and this likely resulted from embryo narcosis. Similarly, in exposures to South Louisiana Sweet WAFs, reproductive failure was increased in the 30% and 100%-WAFs (but oddly not in 50%-WAF). Population growth projections over three generations indicated elevated naupliar production in the 10%, 30% and 50% -NIST WAF, while reduced projections in the 100% -NIST WAF and all South Louisiana Sweet WAFs, relative to controls. Naupliar projections were influenced by differences in overall copepod development, and by slight differences in mean embryo hatching success and viable offspring production. The population growth model presented here was used only as a tool to predict potential population level outcomes. We do not imply that low levels of NIST WAF will provide an ecological advantage to exposed copepod populations. In fact, in this study faster copepod development in exposures to NIST WAFs did not result in higher viable production.

We also evaluated the effect of light regime (UV vs. fluorescent) on NIST WAF (10%, 30% and 50%) acute and chronic toxicity. Relatively short exposures (< 2 w) to fairly low NIST WAF concentrations (i.e., 30%- and 50%-WAF) in combination with low UV levels (i.e., several fold lower than sunlight UV levels) were sufficient to cause negative impacts on nauplius survival and development. Even though UV alone increased control nauplius mortality by 15% relative to non-UV controls, the UV WAF combination resulted in enhanced nauplius mortality, particularly in the 50%-WAF treatment. Furthermore, nauplius exposed to 30% and 50%-WAF in the presence of UV, showed significant delays in development into the copepodite stage. These results are likely related to UV-induced photoactivation of crude oil WAF (Lee 2003). Numerous studies have described the photoactivation of certain PAHs (anthracene, benzo(a)pyrene, fluoranthene, pyrene, benzo[a]anthracene and dibenzothiophene) by UV (Ankley et al. 1997; Lee 2003; Newsted and Giesy 1987; Pettelier et al. 1997). PAH photo-toxicity results from energy absorption into PAH molecules generating super-reactive molecules; these highly oxidizing free radicals can damage biological macromolecules such as DNA (Newsted and Giesy 1987). In exposures, WAFs from various crude oil sources and under UV conditions, Pettelier et al. (1997) found a several fold increase in sublethal toxicity to bivalve

embryos, *Mulinia lateralis*, compared to toxicity under fluorescent light. Fluoranthene was the only photoactive PAH detected in 100%-NIST WAF. However, we do not know if this PAH at a concentration of 14.15± 2.49 µg/L, is solely responsible for the observed UV-mediated acute and subacute toxicity. The phototoxic PAH, dibenzothiophene (Lee 2003), is one of the major PAHs found in NIST crude oil at a concentration of 33± 2 µg/g. This PAH, however, was excluded from our WAF chemical analysis because we had no reference internal GC-MS standard for it. Perhaps, the combined phototoxicity of fluoranthene and dibenzothiophene could be responsible for the effects presented here, but follow-up studies would be required to answer this question.

Conclusions and Recommendations:

1. The Microplate-Based Copepod Lifecycle Bioassay (ASTM 2004), when coupled with the NIST Crude Oil reference standard, functions with high precision, high control repeatability, and consistent WAF constituent generation. It also allows easy control and evaluation of UV-mediated toxicity which can be significant. Bioassay costs to managers are less than for similar lifecycle bioassays with macrofauna (e.g., shrimps, crabs, fishes), and waste disposal amounts/costs are orders of magnitude lower. The scale of this chronic testing approach allows benchmarking of multiple bioassays against a common NIST standard. Benchmarking provides a unique and useful "positive" reference dataset that can be expanded with additional tests of other crude/fuel oil WAFs. All of this can be accomplished up to and including the population-level of biological organization.
2. This study showed that high WAF concentrations of NIST and South Louisiana Sweet crude oil pose an acute and chronic risk to the early life stages of the copepod *A. tenuiremis* -- effects that could potentially be exacerbated by UV radiation. Copepods comprise 10 to 40% of the meiobenthic fauna (Coull 1999) and constitute an important component of the benthic community in coastal and estuarine areas (Coull 1999; Coull and Chandler 1992). Crude oil effects on meiobenthic copepods, such as reduced naupliar survival and therefore copepod abundance, could reduce the food available to higher trophic levels (Street et al. 1998).
3. Further studies should focus on the link between subcellular, organism-level, and long term population consequences of chronic exposures to *post-remediation* crude oil-WAF exposure conditions. The frequently observed developmental accelerations (at low-level WAF) and decelerations (at higher % WAF) suggest a hydrocarbon influence on endocrine-mediated processes if not on endocrine receptors *per se*.
4. This study was intended as a one-year "proof of concept" project to assess the utility of the copepod microplate lifecycle bioassay for measuring sublethal toxicities of crude oil-WAF. That utility is obvious from the data presented. The utility of the NIST crude oil standard as a positive control or benchmark in this microbioassay is a major "added value" that worked unexpectedly well. The cost of using a NIST standard for larger-than-meiobenthic sized test fauna would be prohibitive; for this microplate-based bioassay, NIST standards cost approximately $120 per lifecycle test since the total test volume of WAF required was only about 10 mL. The remaining WAF was of sufficient volume for chemical analyses.

6.0 Technology Transfer

The microplate bioassay method was finally adopted as an ASTM Standard in October 2004. This study is a direct adaptation of that method for crude oil testing. We are presently involved in a multi-laboratory validation exercise for the Organization for Economic Cooperation and Development (OECD) and USEPA that should lead to adoption of the bioassay as a global standard for regulatory uses. The WAF crude oil testing approach developed in this project will prove a useful extension/application for this international effort. Our corporate sponsor who assisted us in obtaining the original South Louisiana Sweet crude oil sample was Research Planning Inc. (Dr. Jacqueline Michel). All reports and coming publications will be copied to them.

7.0 Achievement and Dissemination

7.1 Publications

Bejarano AC, GT Chandler, L He, TL Cary and JL. Ferry. 2005. Risk assessment of low-level NIST petroleum crude oil water accommodated fractions (WAFs) for a meiobethic copepod: Further application of a copepod-based Leslie (Lefkovitch) matrix modeling approach. Environmental Toxicology and Chemistry. (*In Review.*).

Bejarano AC, GT Chandler, L He and BC Coull. 2005. Lethal and sub lethal effects of Louisiana sweet petroleum crude oil water accommodated fractions (WAFs) on a meiobenthic copepod in microplate bioassay. J. Experimental Marine Biology and Ecology (*In Press*)

7.2 Presentations

Bejarano AC, TL Cary, GT Chandler. 2004. Assessing the effects of crude-oil WSF on a meiobenthic copepod using a rapid full-life cycle bioassay. 4th SETAC World Congress. 14-18 Nov. Portland OR, USA. (Poster).

Bejarano AC, GT Chandler, L. He. 2005. Utility of a NIST crude oil water accommodated fraction (WAF) as a benchmark for copepod reproductive and population-level toxicity. Submitted abstract: SETAC North America 26th Annual Meeting. 13-17 Nov. Baltimore, Maryland (Oral; Invited).

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Bejarano, A.C., Chandler, G.T. and Decho, A.W. 2005. Influence of natural dissolved organic matter (DOM) on acute and chronic toxicity of the pesticides chlorothalonil, chlorpyrifos and fipronil to the meiobenthic estuarine copepod Amphiascus tenuiremis. Journal of Experimental Marine Biology and Ecology 321: 43-57.

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Figure 1. Experimental set-up of the full-life cycle bioassay exposing the copepod *Amphiascus tenuiremis* to NIST crude oil- water soluble fraction (WAF) dilutions and seawater control (0%-WAF) under fluorescent (non-UV) and ultraviolet (UV) regimes. **N**= nauplius, **C** = copepodite, **A** = adult copepod.

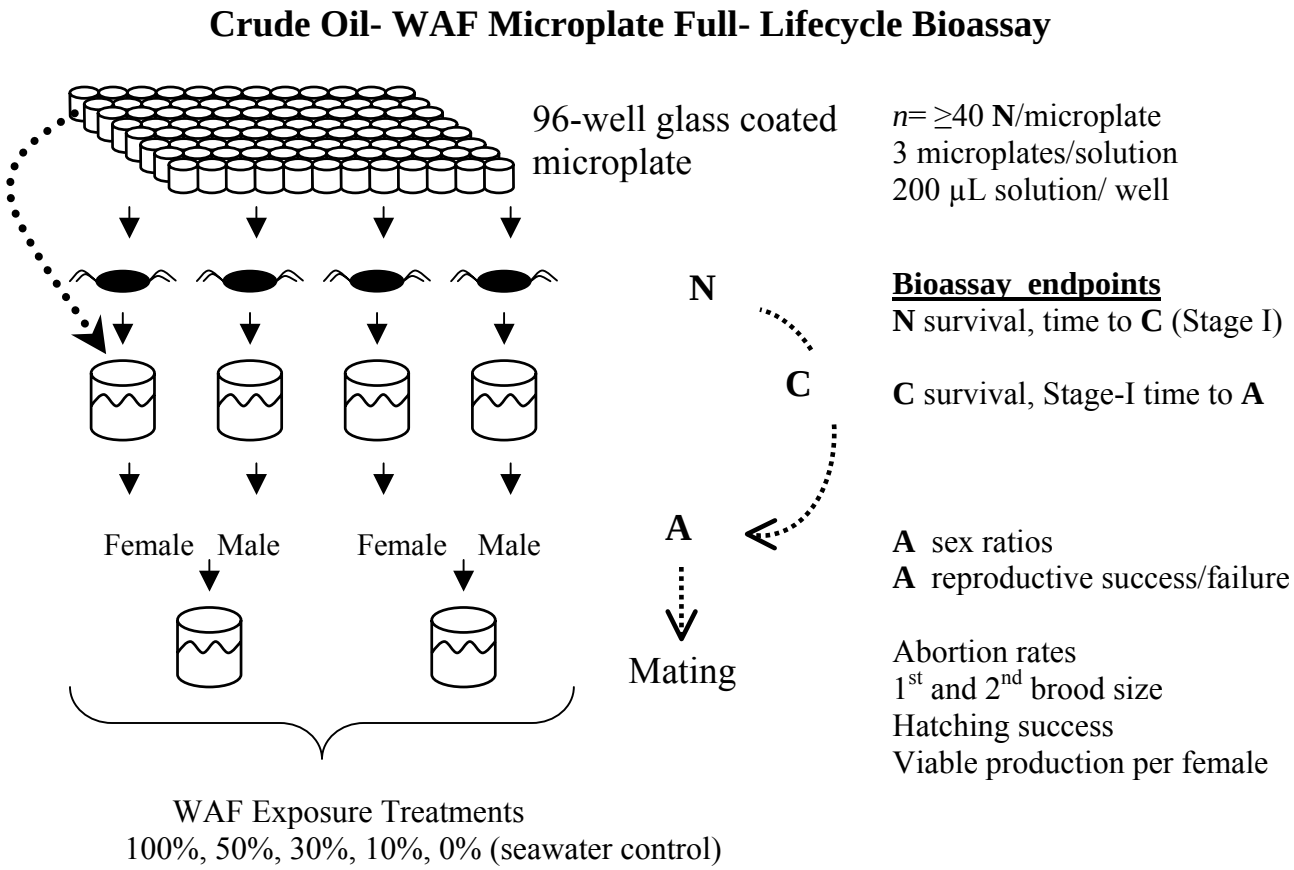


Figure 2. Nauplius-to- Stage I copepodite and Stage I copepodite-to-adult survival in individuals exposed to NIST and South Louisiana Sweet crude oil-water accommodated fractions (WAF) serial dilutions and control (0%). * Represents significant difference vs. control (0%-WAF)

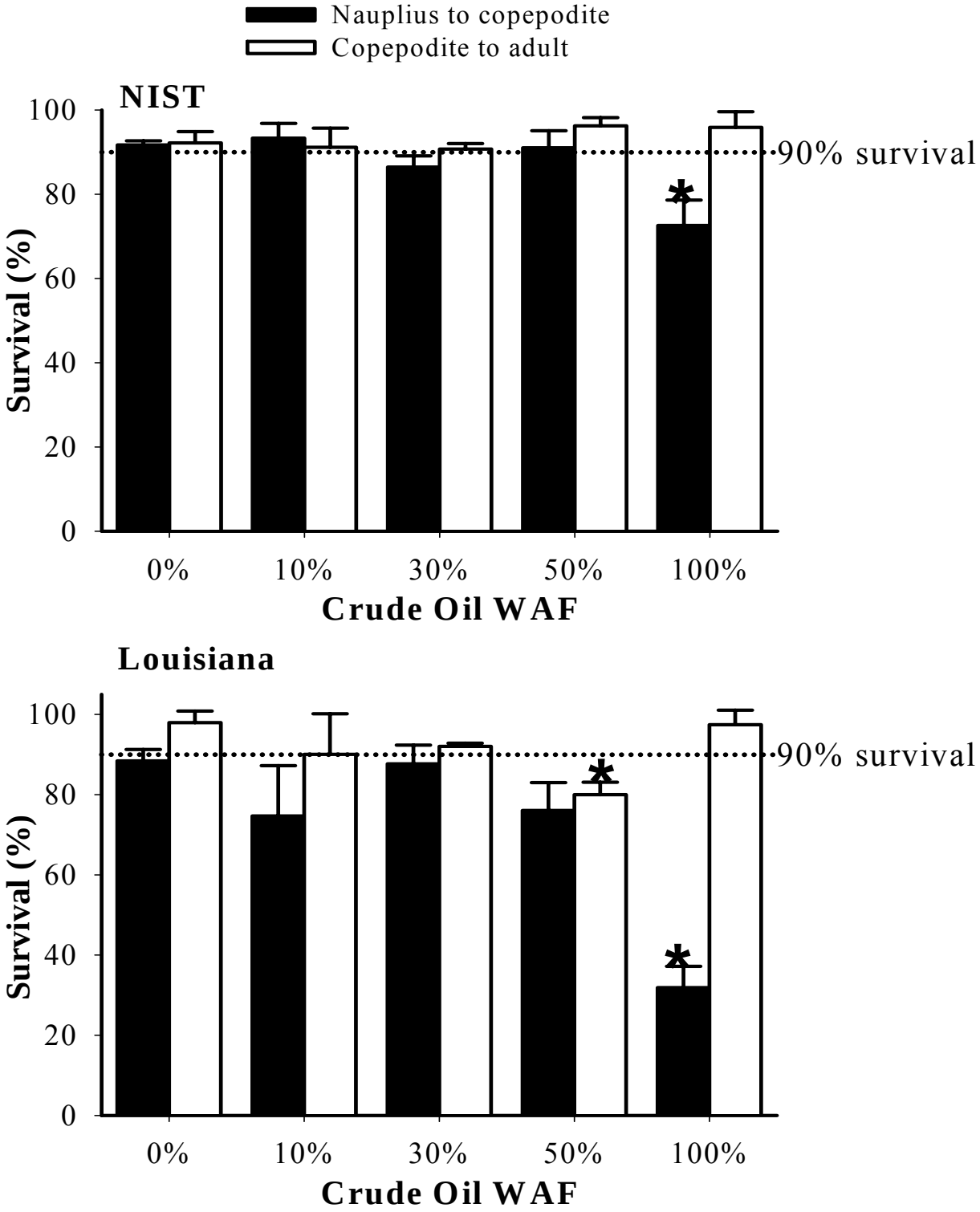


Figure 3. (**A**) Estimated development curves of *Amphiascus tenuiremis* nauplius-to-Stage I copepodite or (**B**) Stage I copepodite to female (F) or male (M) (*n*=124-143/treatment) copepods chronically exposed to NIST crude oil-water accommodated fraction (WAF) serial dilutions. Development into F in 10%, 30% and 50%-WAF, and into M in 10% and 50%-WAF are not shown. P-values represent regression differences compared to controls, and underlined values are significant. C1 = Stage I Copepodite. 0-WAF = clean seawater control.

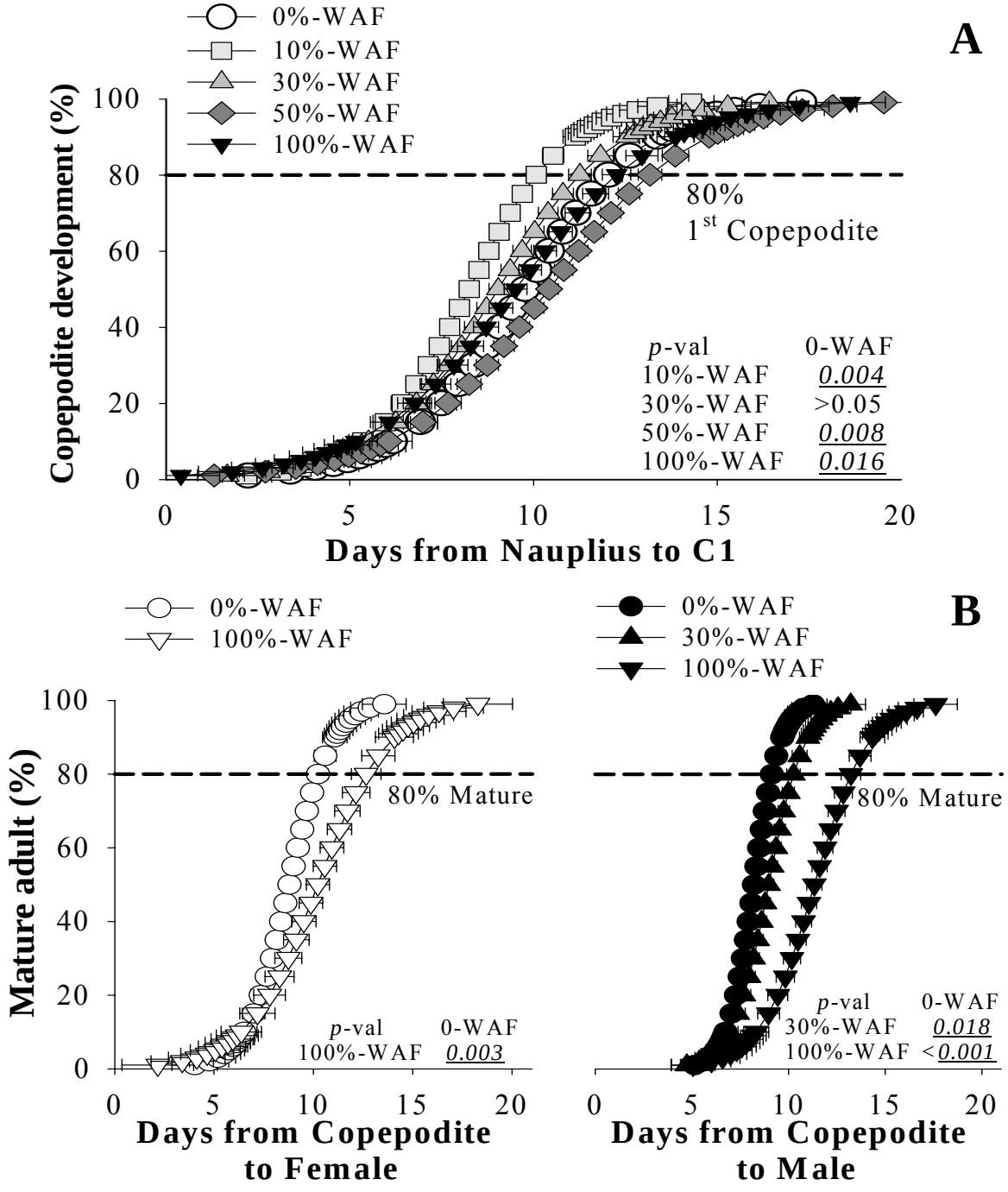


Figure 4. (**A**) Estimated development curves of *Amphiascus tenuiremis* nauplius-to-Stage I copepodite (*n*= 31-98 nauplii/ treatment) or (**B**) Stage I copepodite to female (F) or male (M) (*n*=30-96 copepodites/ treatment) copepods chronically exposed to NIST crude oil-water accommodated fraction (WAF) serial dilutions. Development into F and M in 30% and 100%-WAF are not shown. P-values represent regression differences compared to controls, underlined values are significant. 0-WAF = clean seawater control.

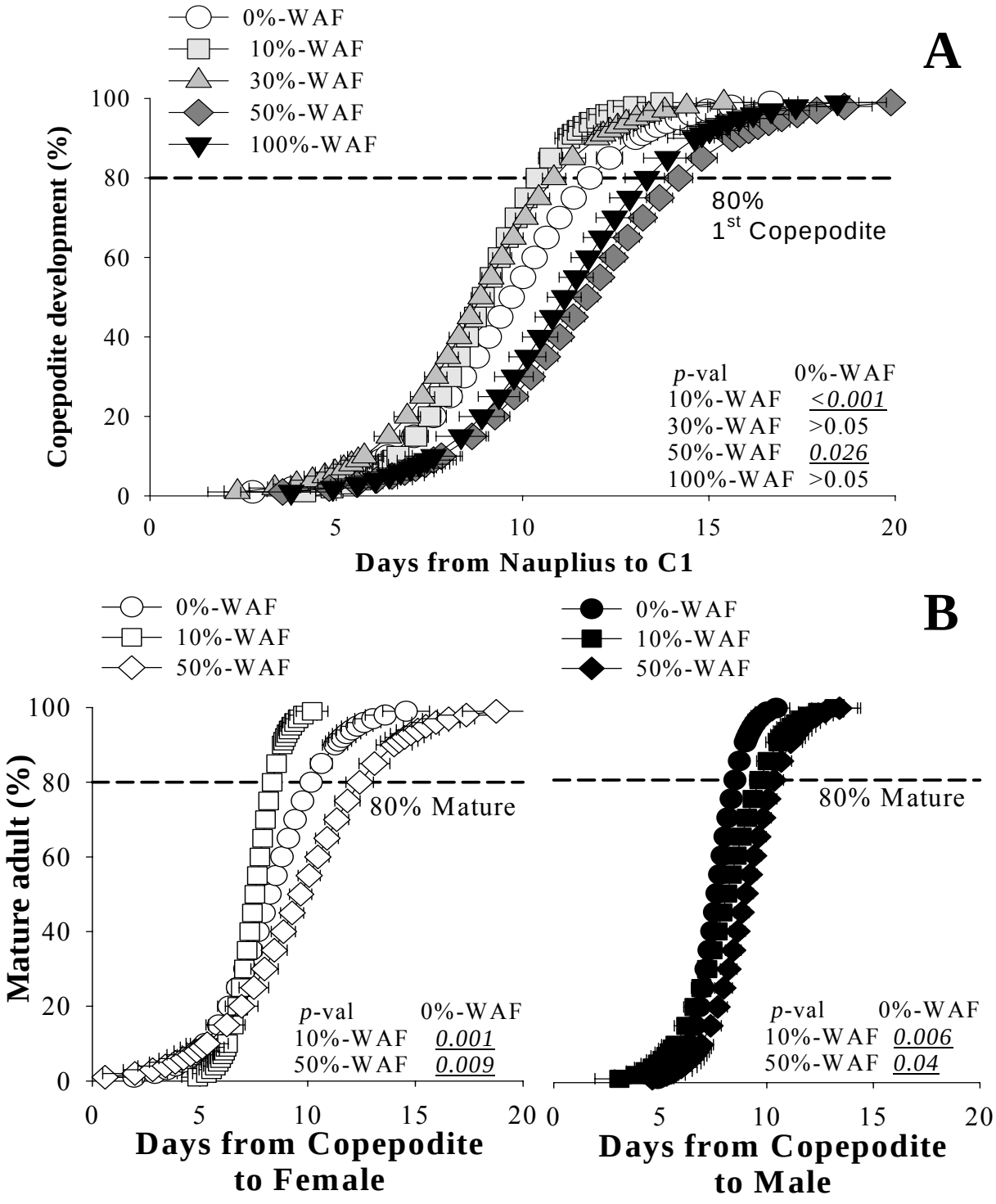


Figure 5. Leslie model projected naupliar production through three generations in *Amphiascus tenuiremis* exposed to NIST and South Louisiana Sweet crude oil-water accommodated fractions (WAF). Empirical data were used in this model. WAF included 10%, 30%, 50% and 100%-strength WAF and seawater control (0%).

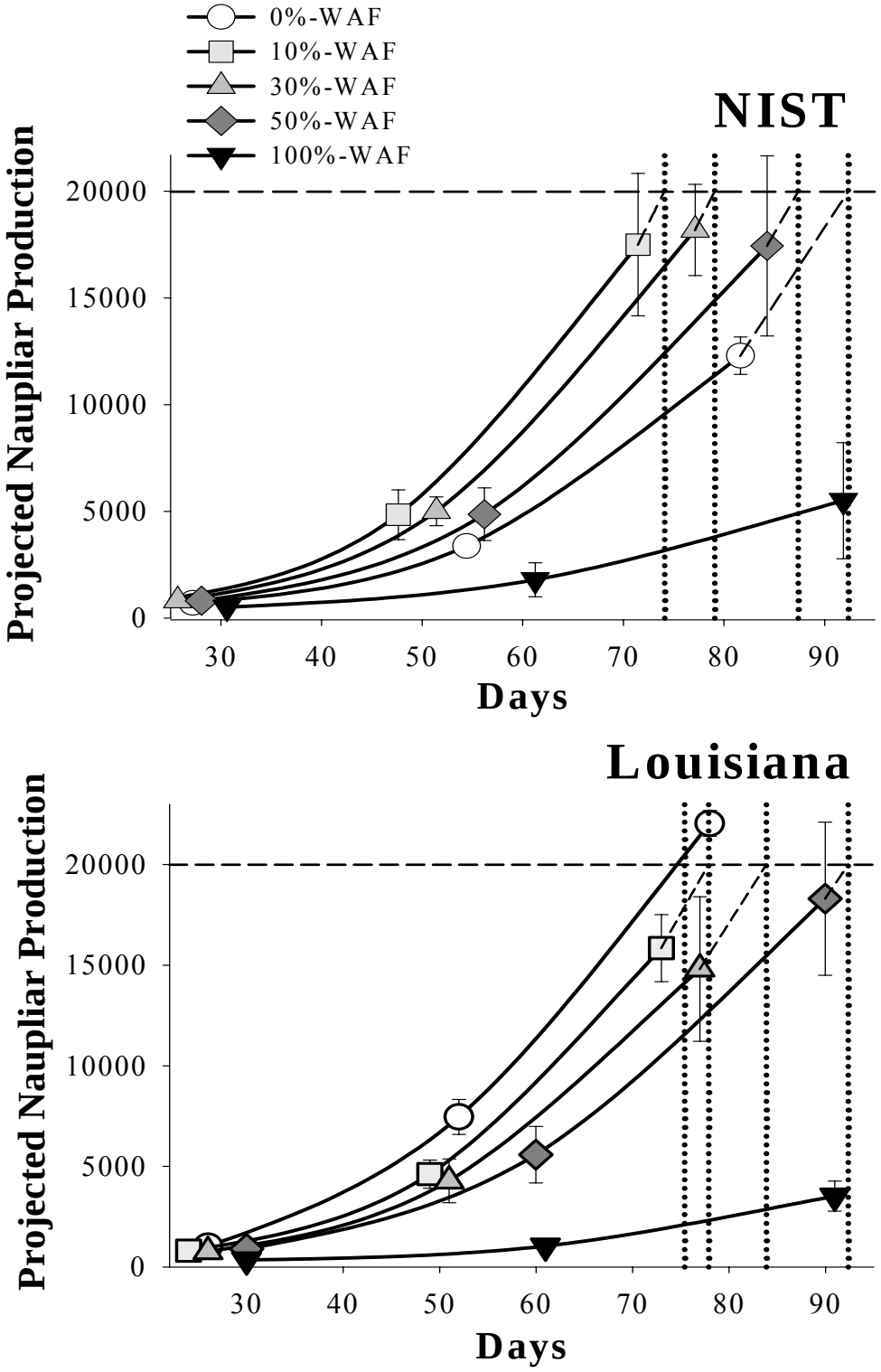


Figure 6. Naupliar mortality in exposures to NIST crude oil-water accommodated fractions (WAF) under fluorescent (non-uv) and UV regimes. Treatments included 10%, 30% and 50%-WAF and seawater control (0%). Long dashed lines (_ _ _) represent predicted Probit curve, while (♦♦♦♦♦) represents the estimated median lethality time (LT50).

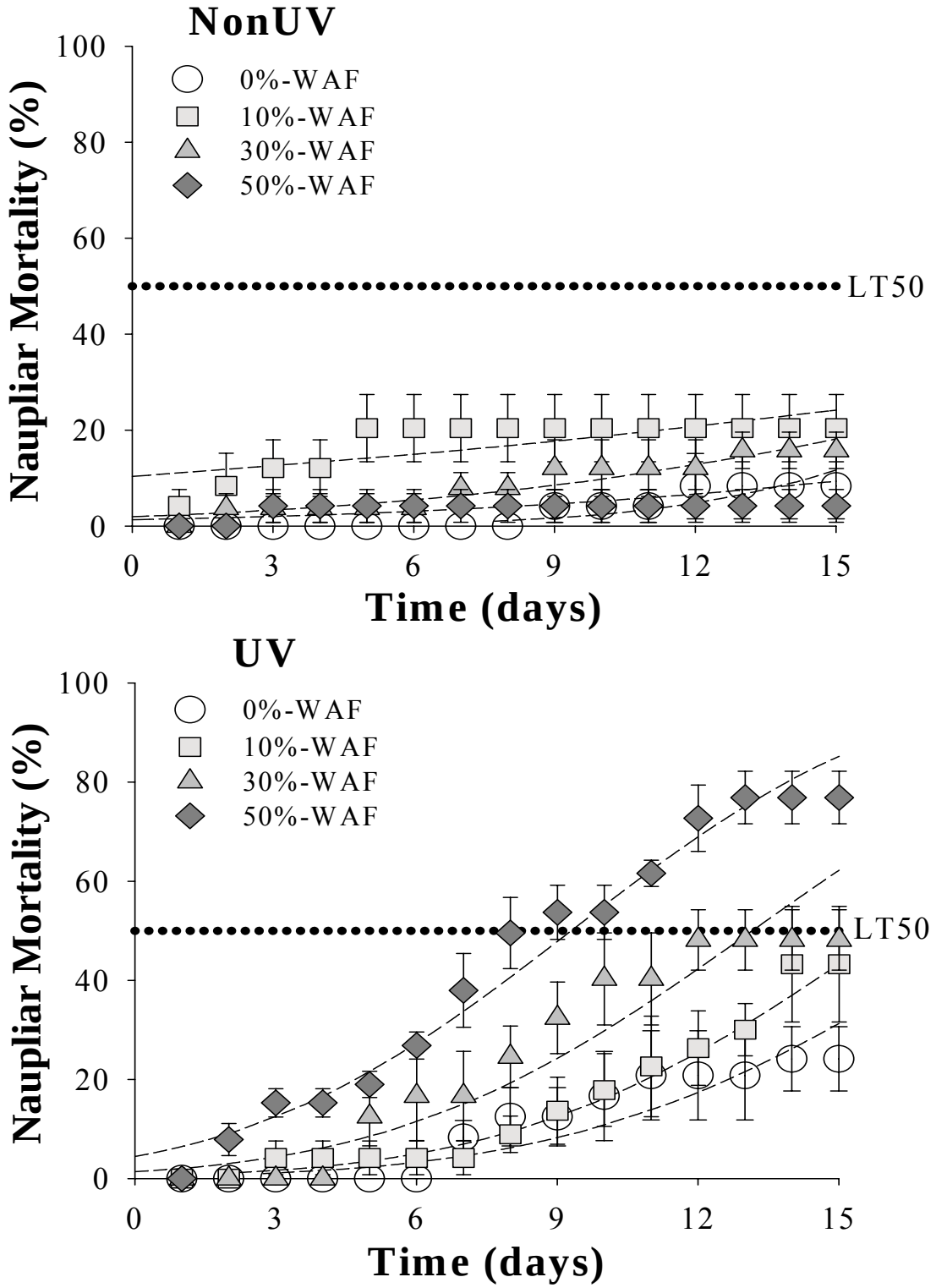


Figure 7. Estimated nauplius-to-stage I copepodite development curves of *Amphiascus tenuiremis* chronically exposed to NIST crude oil-water accommodated fractions (WAF) serial dilutions under fluorescent (Non UV) and UV regimes. P-values represent within treatment regression differences based on slopes and intercepts. Non UV and UV-curves for the 10%-WAF are not shown. C1 = Stage I copepodite.

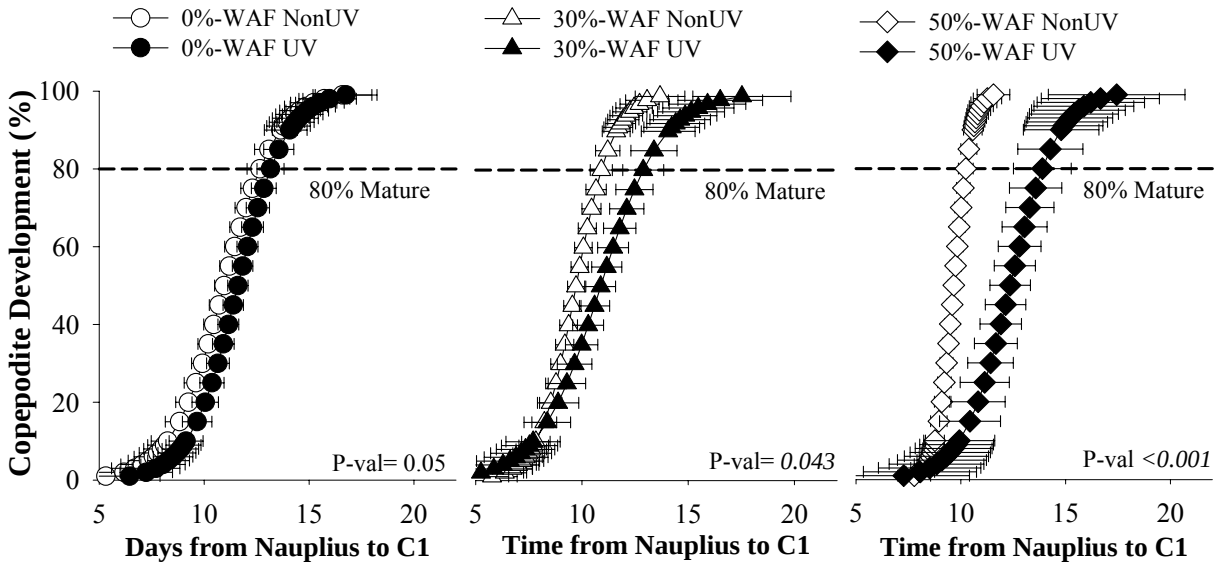
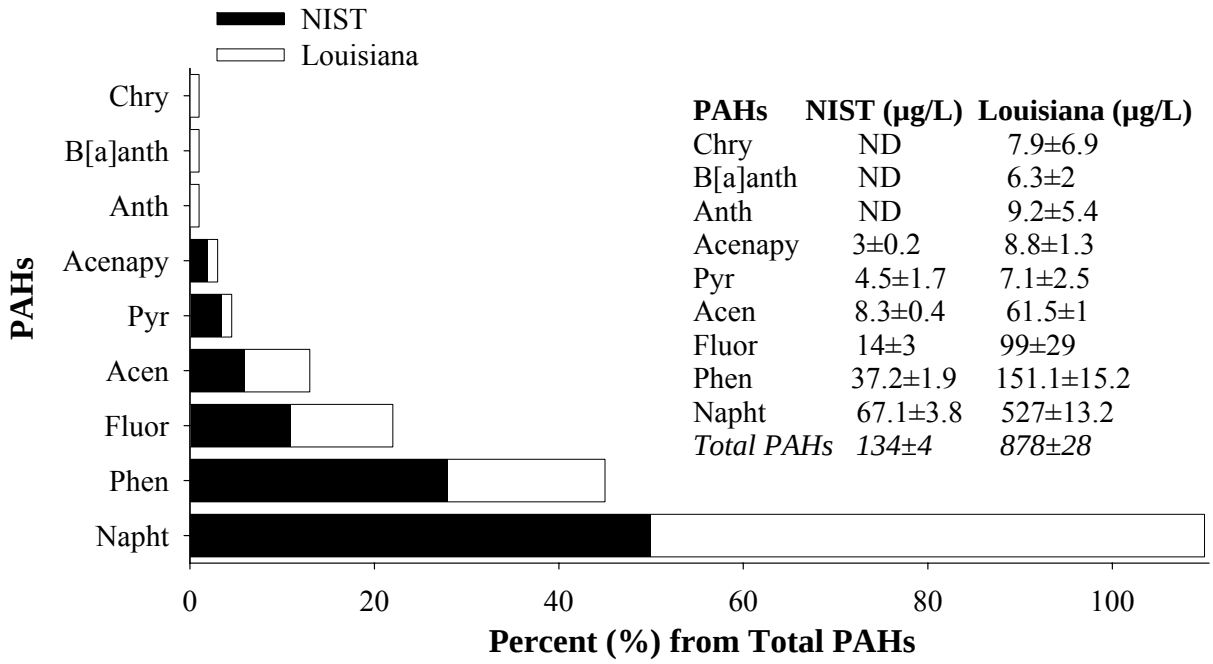


Figure 8. PAH analysis of freshly extracted 100%-water accommodated fraction (WAF) from NIST and South Louisiana Sweet Crude Oil, using EPA SW-846 Method 3510C (n=3 replicates per WAF). PAH analytes include: Acenapthene (Acena), Acenaphthylene (Acenapy), Anthracene (Anth), Benzo [a] anthracene (B[a]anth), Chrysene (Chry), Fluoranthene (Fluor), Naphthalene (Napht), Phenanthrene (Phen), Pyrene (Pyr). PAH analyzed but non-detected or below instrument detection limits included: Benzo [b] fluoranthene, Benzo [k] fluoranthene, Benzo [a] pyrene, Benzo [g, h, i] perylene, Dibenzo [a, h] anthracene, Fluorene and Ideno [1,2,3-c, d] pyrene.



PAHs	NIST (µg/L)	Louisiana (µg/L)
Chry	ND	7.9±6.9
B[a]anth	ND	6.3±2
Anth	ND	9.2±5.4
Acenapy	3±0.2	8.8±1.3
Pyr	4.5±1.7	7.1±2.5
Acen	8.3±0.4	61.5±1
Fluor	14±3	99±29
Phen	37.2±1.9	151.1±15.2
Napht	67.1±3.8	527±13.2
Total PAHs	*134±4*	*878±28*

Table 1. Representative sample of previous oil-pollution studies on meiofauna where copepod effects/non-effects were noted. In the site column, L = Laboratory, M = Mesocosm, F = Descriptive Field Study, F, E = Experimental Field Study. (Abstracted from Coull and Chandler 1992, Review).

POLLUTANT SOURCE/ TYPE	SITE	TAXA STUDIED	EFFECTS	AUTHORS
Crude oil, water-surface applied; oil inhibition of O_2 diffusion	L	*Tigriopus californicus* (Copepod)	1.5-mm crude layer caused **100% death in 3 d**; crude encased by dialysis bag caused 100% **death at 7 d**; aeration delayed death 2-4 d.	Kontogiannis & Barnett 1973
Louisiana crude oil WAF; 0, 50, 75% dilutions	L	*Nicocra affinis* (Copepod)	**Brood size reduced 43%** in all WSF treatments	Ustach 1979
Aromatic hydrocarbons- extracted from 2 crude oils- phytoplankton exposed to hydrocarbon fed copepods	L	*Tigriopus brevicornis* (Copepod)	**Consumption of algae by copepods was reduced** when algae contaminated by aromatic hydrocarbons	LaCaze & Ducreux 1987
Crude oil added to microcosms at 10 & 100 g oil/Kg sandy sediments	M	Nematodes & copepods	Nematode densities increased, **copepod decreased in 1st 2 mo**; nematode diversity decreased in high-oil microcosms after 3 mo; no change in low oil microcosm	Boucher et al . 1981
No. 2 fuel oil WAF; Chronic 4-12 mo. exposure of MERL mesocosms or 90 or 190 ppb in water column; 140 mg/Kg in sediments	M	Major taxa	190 ppb: 38% reduction of total meiofuna; **57% copepod decrease**; 257% foraminifera increase; 90 ppb: **35% copepod decrease**	Oviatt et al. 1982
[Same as above; Oviatt et al. 1982]	M	Copepod species	**Initial mortality & depressed population growth of 2 spp. Over 7 mo.**	Stacey & Marcotte 1987
South Louisiana Sweet crude oil; Surface applied to salt march at 2 L oil/M^2 marsh; 44 mg oil/Kg dry sediment, Louisiana, USA.	F, E	Major taxa, copepod species	No oil- induced mortality; total meiofauna increased in oiled sediments; **Rare copepods disappeared**	Fleeger & Chandler 1983
Crude-oil/azoic sediment mixtures (0, 1.3, 3.8 g oil/Kg dry sediments); Recolonization in *Spartina* salt march, Louisiana, USA.	F, E	Major taxa, copepod species	Nematode densities depressed in highest oil treatment; **depressed copepod colonization for 30 d**; one copepod species increased in high oil by 60 d	Decker & Fleeger 1984
AMOCO Cadiz crude oil spill, 3 sandy beaches, Brittany, France	F	Copepod species	Maximum oil period delayed/depressed spring reproductive peak**; gravids & copepodites often missing**; complete recovery in 2-3 yr	Bodin 1991

Table 2. Reproductive endpoints of *Amphiascus tenuiremis* chronically exposed to NIST crude oil-water accommodated fraction (WAF) serial dilutions. * and p-values (underlined) represent statistical difference vs. control response. N= nauplius, F= female. "n" = number of mating pairs per treatment.

WAF treatment (n = 161)	Reproductive failure (%)	Embryo hatching (%)	Total viable	N to 2nd brood extrusion (days)
0% (28)	14.7± 3.54	87.0± 19.38	12.9±4.12	27.2± 2.03
10% (45)	20.0± 5.44	92.3± 15.84	14.5±4.29	23.8± 1.63* (<*0.0001*)
30% (41)	12.1± 6.61	95.4± 7.87	14.5± 4.68	25.7± 1.97* (*0.0086*)
50% (33)	22.1± 6.61	88.9± 11.52	15.4± 4.45	28.1± 2.28
100% (14)	36.7± 12.47	69.9± 21.26* (*0.0035*)	11.7± 4.74	30.7± 2.97* (*0.001*)

Table 3. Reproductive endpoints of *Amphiascus tenuiremis* chronically exposed to South Louisiana Sweet crude oil-water accommodated fraction (WAF) serial dilutions. * and p-values represent statistical difference vs. control response. N= nauplius, F= female. 'n' = number of mating pairs per treatment.

WAF treatment (n=110)	Reproductive failure (%)	Embryo hatching (%)	Total viable production	N to 2nd brood extrusion (days)
0% (33)	3.33± 4.71	99.47± 3.03	18.18± 3.55	26.06± 2.32
10% (29)	23.7± 12.08	99.79± 0.95	16.09± 4.51	24.3± 1.98* (*0.005*)
30% (22)	32.8± 8.43* (*0.03*)	92.11± 12.39* (*0.02*)	15.47± 5.19	25.72± 2.26
50% (19)	11.43± 8.41	96.08± 6.32	15.41± 2.75	30.03± 1.71* (*<0.0001*)
100% (7)	44.44± 7.86* (*0.015*)	93.06± 7.22	15± 1.22	30.45± 2.44* (*0.0011*)

Table 4. Summary of survival, developmental and reproductive effects of NIST and South Louisiana Sweet crude oil-water accommodated fractions (WAF) on *Amphiascus tenuiremis*. Treatments include 10%, 30%, 50% and 100% WSF and a seawater control (0%-WAF). Only endpoints where differences were found vs. control are included in the table. N= nauplius, C= copepodite, F= adult female and M= adult male.

Bioassay endpoints	Effect relative to control	NIST WAF	Louisiana WAF
N mortality	Increased	100%	100%
C mortlaity	Increased	NONE	50%
N to C development	Delayed	50%, 100%	50%
	Enhanced	10%	10%
C to F development	Delayed	100%	50%
	Enhanced	NONE	10%
C to M development	Delayed	30%, 100%	50%
	Enhanced	NONE	10%
Reproductive failure	Increased	NONE	30%, 100%
Hatching success	Reduced	100%	30%
Total life time	Shorter	10%	10%
	Longer	30%, 100%	50%, 100%
Estimated population size	Increased	10%, 30%, 50%	NONE
	Reduced	100%	10%, 30%, 50%, 100%