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RISK ASSESSMENT OF THE NATIONAL INSTITUTE OF STANDARDS AND TECHNOLOGY PETROLEUM CRUDE OIL STANDARD WATER ACCOMMODATED FRACTION: FURTHER APPLICATION OF A COPEPOD-BASED, FULL LIFE-CYCLE BIOASSAY

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Abstract—The U.S. National Institute of Standards and Technology (NIST) petroleum crude oil was used to generate NIST water-accommodated hydrocarbon fractions (WAFs) for standardized assessment of crude oil effects on the copepod *Amphiascus tenuiremis*. Effects were assessed using a 96-well microplate, full life-cycle test. Briefly, nauplii (age, 24 h) were reared individually to adults ($n \geq 120$ nauplii/treatment) in microplate wells containing 200 μl of treatment solution (seawater control [0%] or 10, 30, 50, or 100% NIST-WAF). Nauplii were monitored through development to adulthood, and mature virgin male:female pairs mated in wells containing original treatments (<30 d). A second bioassay using 0, 10, 30, and 50% WAFs ($n \geq 60$ nauplii/treatment) was conducted to assess the effects of ultraviolet (UV) light on naupliar endpoints (<16 d). In the first experiment, nauplius-to-copepodite survival in exposures to 100% WAF was 27% ± 6% lower than in controls (92% ± 1%), but copepodite-to-adult survival was greater than 90% across all treatments. Analysis of development curves showed that nauplii in the 10% WAF developed into copepodites 25% faster, whereas nauplii in the 50 and 100% WAFs developed 17% slower, than controls. Copepodite development into male and female copepods was significantly delayed (2 and 4 d, respectively) in the 100% WAF compared to controls. Although none of the WAF exposures had significant effects on fertilization success or total viable production ($p > 0.05$), embryo hatching in the 100% WAF was significantly less (70.0% ± 21.2%) than that in controls (87.0% ± 19.4%). Results from the UV bioassay showed that relatively short exposures (<14 d) to 30 and 50% WAFs in the presence of UV light caused negative effects on copepod survival and development. Naupliar-stage survival and developmental endpoints were the most sensitive indicators of exposure to the NIST crude oil WAF.

Keywords—Meiobenthic copepod Life-cycle bioassay Crude oil water-accommodated fraction Sublethal toxicity Ultraviolet-mediated toxicity

INTRODUCTION

Petroleum crude oil inputs into marine environments result from natural seeps, extraction, transportation, and consumption (i.e., urban runoff and operational discharges) of petroleum [1]. Although oil spills from transportation vessels represent less than 2% of the crude oil discharge into U.S. waters [1], these have environmental significance because of their acute effects and potentially long-lasting population-, community-, and ecosystem-level impacts. Crude oil is composed of a myriad of chemicals, including saturated (i.e., alkanes and cycloalkanes) and unsaturated (i.e., oleofines) compounds, monoaromatic (i.e., benzene, toluene, ethylbenzene, and xylene [known as BTEX]) and polycyclic aromatic hydrocarbons (PAHs) [2]. The most toxic fractions are the highly soluble (low molecular wt, C_{10}; i.e., naphthalenes) and moderately soluble (C_{10}–C_{20}) nonaromatic fractions [2]. Once released into the aquatic environment, crude oil partitions into volatile, water-soluble (i.e., water-accommodated) and insoluble fractions, with the water-accommodated fraction (WAF) and oil residue representing the most persistent fractions and important sources of exposure to aquatic organisms [3,4]. Oil residue in sediments serves as a WAF source to pore and surface water, where soluble oil contaminants may pose long-term hazardous conditions to benthic communities. Studies have shown that crude oil and other petrochemicals have deleterious effects on benthic estuarine invertebrates, including polychaetes [5], penaeid shrimp [6], larval decapods [7], and amphipods [8]. Early studies [9] with juvenile shrimp (*Crangon crangon*) showed that crude oil WAF not only increased juvenile mortality, but also caused a reduction in respiration and growth rates. Other studies also have shown that crustaceans are particularly susceptible to sediment- and pore water–associated crude oil contamination [10–12]. In microcosm studies of Louisiana salt-marsh meiobenthic community response to sediments spiked with diesel fuel, Carman et al. [13] found that diesel hydrocarbon contamination negatively affected estuarine microcrustaceans (copepods, ostracods, and early copepod life stages; i.e., nauplii), primarily by reducing copepod species richness and ostracod and naupliar abundance. Many other studies also have observed high copepod sensitivities to crude oil (see, e.g., [11,12]), but typically under the worst-case exposure conditions immediately following oil-spill events.

In the present study, we explored the utility of a full life-cycle test (American Society for Testing and Materials [ASTM] [14]) with the meiobenthic harpacticoid copepod *Amphiascus tenuiremis* for assessment of the risk of petroleum crude oil WAFs on development, reproduction, and potential population growth. The well-characterized U.S. National Institute of Standards and Technology (NIST) standard crude oil was used to generate a standard WAF for comparative assessment of crude oil effects using this crustacean model. The NIST standard crude oil, though expensive, can be used as a

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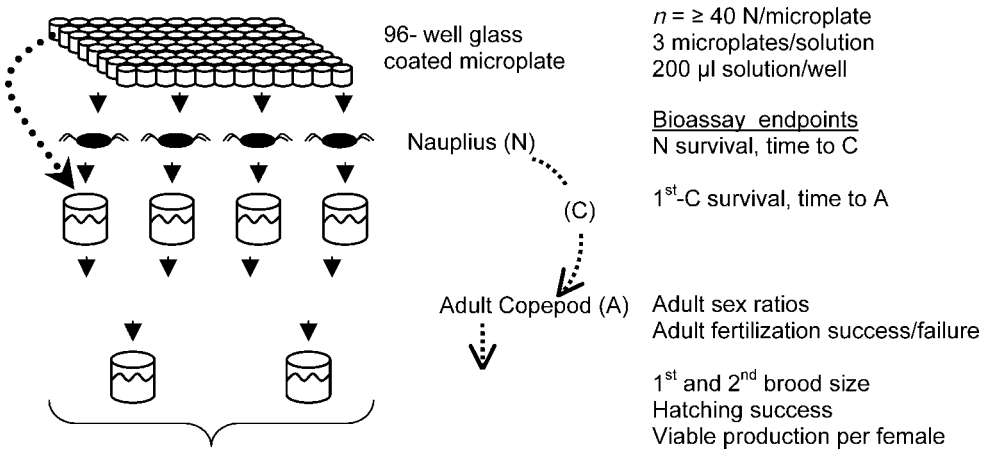

Fig. 1. Experimental setup of the full life-cycle bioassay exposing the copepod *Amphiascus tenuiremis* to National Institute of Standards and Technology (NIST) crude oil water-accommodated fraction (WAF) dilutions and seawater control (0% WAF) under ultraviolet (UV) and fluorescent (non-UV) regimes. A = adult copepod; C = copepodite; N = nauplius.

source for WAF in ASTM E2317-04, because this bioassay requires low-milliliter quantities of test solutions.

MATERIALS AND METHODS

Test organism

Amphiascus tenuiremis is an amphi-Atlantic, infaunal, sediment-dwelling, harpacticoid copepod that inhabits muddy inter- and subtidal estuarine sediments from the Baltic and Black seas to the southern Gulf of Mexico [15]. *Amphiascus tenuiremis* has a generation time of 21 d (egg-to-egg) at 20°C in sediments [16] and a life cycle consisting of nauplii (six stages), copepodites (five stages), and sexually dimorphic adults. This copepod has been used in sublethal and developmental toxicity testing for a wide variety of contaminants [17–19], and it is a useful model for life-history and population-level effects from sediment and waterborne contaminants [16–20].

Preparation of crude oil WAF and WAF solutions

Petroleum crude oil standard (i.e., batches of 2-ml, sealed ampoules) was purchased from the National Institute of Standards and Technology (Standard reference material 1582 [21]; NIST, Washington, DC). Major organic constituents in the NIST standard were phenanthrene (101 ± 5 µg/g), fluoranthene (2.5 ± 0.3 µg/g), benzo[*a*]anthracene (3.0 ± 0.3 µg/g), benzo[*a*]pyrene (1.1 ± 0.3 µg/g), perylene (31 ± 3 µg/g), and dibenzothiophene (33 ± 2 µg/g) [20]. Crude oil ampoules were kept refrigerated (4°C) in the dark until used. Crude oil WAFs were prepared following National Research Council recommendations [2]. Briefly, filtered (pore size, 0.22 µm) and fully saturated (dissolved oxygen, >99%) seawater (30‰, 60 ml) and a Teflon®-coated stirring bar (1 cm) were placed in an amber, round, glass flask (250 ml), and 2 ml of NIST crude oil were layered dropwise to the top of the seawater surface. The flask was sealed tight with Teflon-lined screw caps, and headspace air was manually purged through the Teflon septa using a stainless-steel needle attached to a 50-ml Hamilton gas-tight syringe (Fisher, Pittsburgh, PA, USA). The 190-ml headspace was refilled with nitrogen (purity, >99%) to prevent degradation/oxidation of oil. The flask was placed in a refrigerated incubator on a magnetic stirrer plate and the water stirred in the dark for 36 h at 20 ± 1.5°C. Stirring speed was carefully controlled to avoid a large vortex and formation of oil droplets or emulsions. After 36 h, approximately 90% of the WAF was collected by a glass syringe from the bottom of the flask and pooled into a common WAF lot. The WAF was stored at 4°C in 10-ml aliquots in clean glass vials sealed with aluminum foil caps. Water-accommodated hydrocarbon fractions for chronic copepod exposures were made fresh every two weeks.

Water-accommodated fraction dilutions were made in 250-ml beakers by combining appropriate amounts of fully aerated (30‰) seawater and full-strength WAF. The WAF treatments included full strength (100%) WAF as well as 50, 30, and 10% WAF dilutions, with WAF-free seawater used as control (0% WAF).

Crude oil WAF chronic exposures

A 96-well microplate, full life-cycle bioassay following ASTM E-2317-04 [14] was conducted to assess the effects of NIST crude oil WAF on *A. tenuiremis* development and reproduction (Fig. 1). Gravid *A. tenuiremis* were collected from monoculture in clean 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 bottoms over an 18- to 24-h period.

Nauplii were gently pipetted individually into each of 250-µl wells of triplicate, glass-coated, 96-well microplates (Sun-SRI, Duluth, GA, USA). Each microplate ($n = 3$ per treatment) was loaded with at least 40 nauplii haphazardly assigned one each per treatment or control microwell. Excess transfer seawater was removed (>90%) from each well before the addition of 200 µl of control or WAF treatment solutions. Nauplii were monitored daily through the copepodite stage to sexual maturity. On reaching sexual differentiation, virgin male and female copepods were removed from their original wells and mated pairwise in new wells containing their original WAF treatments. Treatment solutions were replaced (water replacement, >90%) every third day throughout the experiment with fresh treatment solutions (dissolved oxygen, >90%) to ensure proper water quality and consistent WAF or control exposures. Water quality (salinity, temperature, dissolved oxygen, and pH) in fresh test solutions was recorded before each water change. Individuals throughout the duration of the experiment were fed every sixth day with 3 µl of a fresh algae mixture

(10^7 cells/ml of 1:1 *Isochrysis galbana*:*Dunaliella tertiolecta*). Covered microplates were held in an incubator (Revco, Asheville, NC, USA) at 25 ± 1°C and a 12:12-h light:dark photoperiod with cool fluorescent lighting.

Survival and development times were recorded daily for individual naupliar and subsequent copepodite stages via Hoffman differential interference contrast inverted stereomicroscopes. Life-cycle endpoints included naupliar survival and development times to stage-1 copepodite, copepodite survival and development times to sexual differentiation, and adult sex ratios. Likewise, each mating pair was monitored daily during the mating period, which lasted up to 9 d postmating, to accommodate potential toxicant-induced delays in reproduction. Reproductive endpoints included fertilization success/failure, hatching success, first and second brood sizes, and total viable offspring production. Fertilization failure was defined as any mating pair unable to produce viable hatchings through two broods over the entire mating period (9 d).

Stage-structured population growth model

Multigeneration 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, USA) [22,23]. A five-stage (i.e., embryo to nauplius, nauplius to copepodite, copepodite to virgin female, and virgin female to gravid female) matrix model was used to project naupliar production through three generations. Projections of naupliar production were modeled based on stage-specific survival rates, the proportion of copepodites developing into virgin females, the proportion of females able to reproduce, and female fecundity (i.e., viable offspring/female) through two broods. Model constraints included demographic stochasticity and an arbitrarily set carrying capacity of 20,000 individuals. Empirical data from each microplate per treatment were used to simulate naupliar production through three generations over 10 separate, 50-simulation runs of the model. Model simulations run/replicated at the microplate level allow subsequent statistical comparisons of model-derived population-growth predictions across WAF treatments and controls [16–18].

Ultraviolet and fluorescent light exposures

A partial life-cycle (i.e., naupliar to copepodite stage only) microplate bioassay was performed to evaluate potential UV-mediated exposure effects when in combination with WAF exposure. A new batch of NIST crude oil WAF was prepared as described above to test the toxicity of 50, 30, and 10% WAFs in the presence/absence of UV irradiation. The experimental setup was performed as described above, with three microplates per treatment loaded with at least 20 nauplii per plate. Exposures were done under dual-fluorescent and UV exposure conditions in an incubator at 25 ± 1°C. Wells were loaded with 200 μl of WAF or control seawater solution, and microplates were haphazardly assigned in triplicate to either cool fluorescent or UV exposure regimes. The incubator was set to accommodate dual sets of lamps. Ultraviolet lamps were placed above and fluorescent lamps below the exposure table. Microplates assigned to the fluorescent-only exposure regime were protected from UV radiation by covering the lids with aluminum foil. Lids from the microplates assigned to the UV regime were removed and replaced with 6- × 5-cm, 2-mm glass lantern slides to allow for infiltration of UV radiation. Both fluorescent and UV light were measured using an actinometer, with UV-A and UV-B measured between 290 and 320 and between 320 and 400 nm, respectively. Endpoints in this partial life-cycle bioassay included naupliar mortality over time and naupliar development rate/success to stage-1 copepodite juvenile. A full life-cycle bioassay could not be conducted because of high UV-linked WAF mortality above the 20% rate allowed under ASTM E2317-04 [14].

Water chemistry analysis

Fresh 100% WAF (100 ml) extracted with methylene chloride was analyzed for PAH analytes (two- to six-ring structures) following U.S. Environmental Protection Agency SW-846 Method 3510C (http://www.epa.gov/epaoswer/hazwaste/test/pdfs/3510c.pdf), with 50 μl of 2-fluorobiphenyl (96%) and *p*-terphenyl-d14 (98% purity; Aldrich®, St. Louis, MO, USA; 200 μg/ml) used as surrogate standards and 50 μl of phenathrene-d_{10} used as an internal standard. The WAF extracts were analyzed using a Varian® (Palo Alto, CA, USA) 3800 gas chromatograph coupled to a Varian Saturn 2000® tandem mass spectrometer with an ion-trap, mass-selective detector system. Injection port and transfer line temperatures were set at 280°C. The gas chromatograph 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 and 80°C, respectively. A fused silica capillary column (length, 30 m; inner diameter, 0.25 mm; film thickness, 0.25 μm; J&W Scientific, Folsom, CA, USA) was used to separate target PAH analytes.

Statistical analysis

Naupliar-to-copepodite and copepodite-to-adult development curves among WAFs and controls were compared using generalized linear interactive modeling (GLiM) [24] via PROC GENMOD in SAS® software (SAS Institute, Cary, NC, USA). All life-cycle bioassay endpoints were tested for normality and homogeneity of variance using the Shapiro-Wilk goodness-of-fit test and Levene test, respectively. Data failing normality were transformed when appropriate. For the full life-cycle microplate bioassay, treatment-specific differences in stage-specific survival, percentage mating success, first and second brood sizes, hatching success, and total viable offspring production were determined by a one-way analysis of variance (PROC GLM; SAS) using the Bonferroni adjustment for multiple pairwise comparisons.

Analysis of UV versus fluorescent light effects on WAF toxicity included naupliar mortality curves over time, in which probit analysis (PROC PROBIT; SAS) was employed to estimate median time to lethality (LT50) values [24]. Naupliar-to-copepodite development curve analysis within WAFs and across light regimes was performed using GLiM as described above. Population-level effects of WAF (full life-cycle bioassay only) were analyzed using population projections for each microplate per WAF treatment and control, with variance estimates computed at the level of individual microplates. 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 $\alpha = 0.05$.

RESULTS

Dilution series exposures

The first full life-cycle bioassay of the NIST petroleum crude oil standard WAF recorded the development of nauplii from 24 h of age through female extrusion of their second

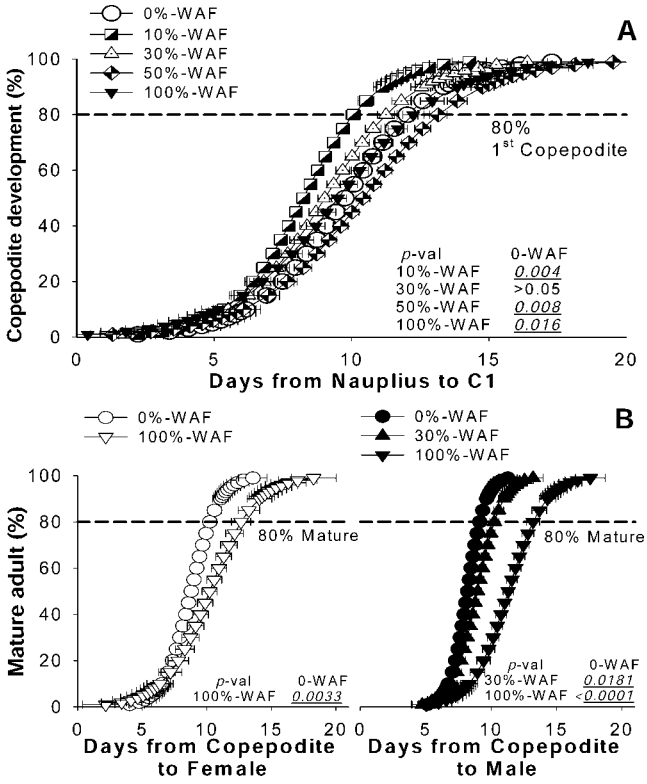

Fig. 2. Estimated (**A**) nauplius-to-first-copepodite and (**B**) copepodite-to-female (F) or -male (M) development curves of *Amphiascus tenuiremis* (n = 124–143 per treatment) chronically exposed to National Institute of Standards and Technology crude oil water-accommodated fraction (WAF) serial dilutions. Development into F in 10, 30, and 50% WAF, and into M in 10 and 50%-WAF are not shown. The p-values represent regression differences relative to controls based on comparisons of slopes and intercepts.

broods (30 d). One of the control microplate replicates for this bioassay was discarded 5 d into the experiment, when the replicate was discovered to have manufacturing defects (multiple leaks). Control naupliar and copepodite survival in the remaining two microplates was greater than 90%. The 100% WAF induced elevated naupliar mortality (27% ± 6%) compared to control values (8% ± 1%; $p = 0.008$), but other WAFs caused nonsignificant mortality. Copepodites were much less sensitive than nauplii and showed greater than 90% survival in all NIST WAF treatments ($p > 0.05$).

Generalized linear interactive modeling of naupliar-to-copepodite development curves predicted 10 to 13 d for 80% naupliar development into the copepodite stage across WAF dilutions and the control. Statistical comparisons of curve slopes and intercepts (Fig. 2A) indicated that most WAF dilutions, except for 30% WAF, yielded developmental curves significantly different from the control. Nauplii exposed to 10% WAF developed, on average, 25% faster than controls, whereas nauplii in the 50% and 100% WAF developed, on average, 17% more slowly than controls. In the copepodite-to-adult development window (Fig. 2B), copepodites exposed to 100% WAF showed a 2- and 4-d delay in development into adult females (i.e., 80% mature at 12.7 ± 0.8 d) and males (i.e., 80% mature at 13.2 ± 0.5 d), respectively, compared to controls (i.e., 80% mature at 9.0 ± 0.3 and 9.1 ± 0.3 d into females and males, respectively). Copepodite-to-male development in all WAF exposures was slower than that in WAF-free controls, but development was significantly slower only in those individuals exposed to 30 and 100% WAF (i.e., 28 and 46% less, respectively, than controls).

Female-to-male sex ratios were variable across microplates and were not significantly different between any WAF treatments and controls. The percentage of females unable to produce at least two viable broods (i.e., fertilization failures) ranged from a low failure rate of 14.7% ± 3.5% (n = 21) in controls to a high of 36.7% ± 12.5% at 100% WAF (n = 9). However, the fertilization failure rate was highly variable within treatments and was not statistically different between any WAF treatment and the control (Table 1). Embryo hatching success over two broods was, on average, 87.0% ± 19.4% in controls, with only the 100% WAF showing significantly reduced success (70% ± 21.2%, $p = 0.004$) compared to controls. Hatching success in the remaining WAF treatments was greater than 89%. None of the WAFs produced statistically significant effects on viable offspring production ($p > 0.05$) compared to controls.

Considering the development times for each of the crucial life stages (i.e., naupliar to stage-1 copepodite, stage-1 copepodite to female, and mating to second brood hatch), all WAF treatments except for the 50% WAF showed significantly different full life-cycle development times (i.e., nauplius to second brood extrusion; $p < 0.05$) compared to controls (Table 1). Individuals exposed to 10 and 30% WAF showed, on average, full life-cycle development times 3 and 1 d shorter, respectively, than controls, whereas individuals exposed to 100% WAF showed, on average, a full life-cycle development time 3.5 d longer than controls.

Table 1. Reproductive endpoints for *Amphiascus tenuiremis* chronically exposed to National Institute of Standards and Technology crude oil water-accommodated fraction (WAF) serial dilutions[a]

WAF treatment	Fertilization failure rate (%)	Embryo hatching (%)	Total viable offspring (n)	Nauplius to second brood extrusion (d)
0% (n = 28)	14.7 ± 3.5	87.0 ± 19.4	12.87 ± 4.12	27.21 ± 2.03
10% (n = 45)	20.0 ± 5.4	92.3 ± 15.8	14.54 ± 4.29	23.82 ± 1.63* ($p < 0.0001$)
30% (n = 41)	12.1 ± 6.7	95.4 ± 7.9	14.47 ± 4.68	25.72 ± 1.97* ($p = 0.009$)
50% (n = 33)	22.1 ± 6.6	88.9 ± 11.5	15.37 ± 4.45	28.1 ± 2.28
100% (n = 14)	36.7 ± 12.5	70.0 ± 21.3* ($p = 0.004$)	11.73 ± 4.74	30.73 ± 2.97* ($p = 0.001$)

[a] The p values and asterisks represent statistical differences versus control. The n value represents the number of mating pairs per treatment.

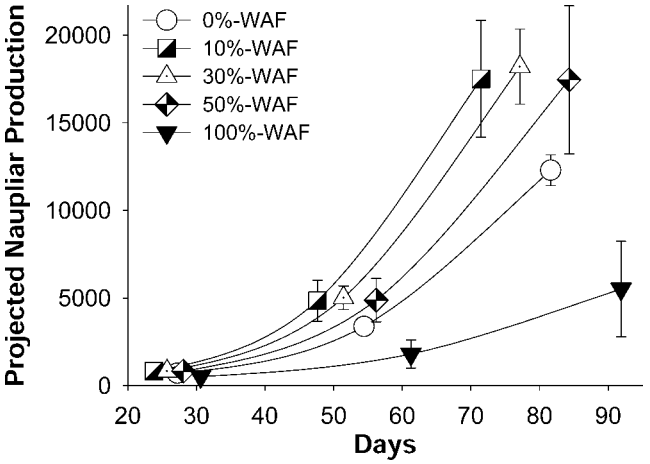

Fig. 3. Leslie-matrix projected naupliar production by *Amphiascus tenuiremis* exposed through three generations to National Institute of Standards and Technology crude oil water-accommodated fractions (WAF).

Stage-structured population growth modeling

Potential population-level responses for controls and WAF life-cycle exposures were predicted using a stage-based, Leslie matrix model [22,23]. Model projections were calculated using empirical endpoint data from each microplate within treatment and empirical generation times (i.e., days from nauplius to second brood extrusion) (Table 1) as time frames for each projected generation (F_1, F_2, and F_3). Because sex ratios were highly variable across treatments and were not significantly influenced by WAF treatments, population projections were performed using the normal 50% virgin female proportion for all WAF treatments and the control. For all WAF treatments versus the control, every WAF naupliar projection through three generations was significantly different ($p < 0.0001$): The 10, 30, and 50% WAFs yielded higher naupliar production projections than the control (Fig. 3), but the 100% WAF yielded 27, 47, and 55% lower projections than the control in F_1, F_2, and F_3, respectively. Naupliar projections in the 10, 30, and 50% WAFs were, on average, 16% higher than controls in F_1 and between 41 and 48% higher than controls in both F_2 and F_3. All these projections were influenced by subtle differences in hatching success and brood sizes between WAFs and controls; the 100% WAF negative response was strongly influenced by elevated naupliar mortality rates and modestly reduced reproductive success. None of these WAF stage-based models would predict population extinction, only population reduction at 100% WAF.

Ultraviolet and fluorescent light exposures

Fluorescent light levels were 376 ± 187 μW/cm², whereas total UV levels (UV-A and UV-B) were 355 ± 24 μW/cm², with UV-B accounting for 13% ± 0.7% of the total light irradiance. Total UV levels in this experiment were fourfold less than natural sunlight UV levels (1,500 μW/cm²) and 18-fold lower than natural sunlight on a cloudless summer day (7,321 μW/cm²) in the southeastern United States.

Naupliar survival to stage-1 copepodite throughout the 16-d partial life-cycle bioassay under the fluorescent-only regime was greater than 80% across WAFs and controls ($p > 0.05$). In contrast, WAF-UV exposures resulted in reduced naupliar survival, from a high of 75.8% ± 11.2% in control microplates to a low of 23.2% ± 9.2% in the highest 50% 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, but not significantly different from that in controls ($p > 0.05$). On average, UV light alone increased naupliar mortality by 15% compared to fluorescent lighting alone. Exposures to WAF in combination with UV light resulted on a dose-dependent naupliar mortality not seen for WAFs in the absence of UV light (Fig. 4). Probit analysis of naupliar mortality data ($p > 0.05$, likelihood ratio chi-square in all cases) estimated a LT50 for UV-exposed individuals of 13.2 d (95% confidence interval, 12.1–14.7 d) for the 30% WAF and of 9.3 d (95% confidence interval, 8.5–10.1 d) for the 50% WAF.

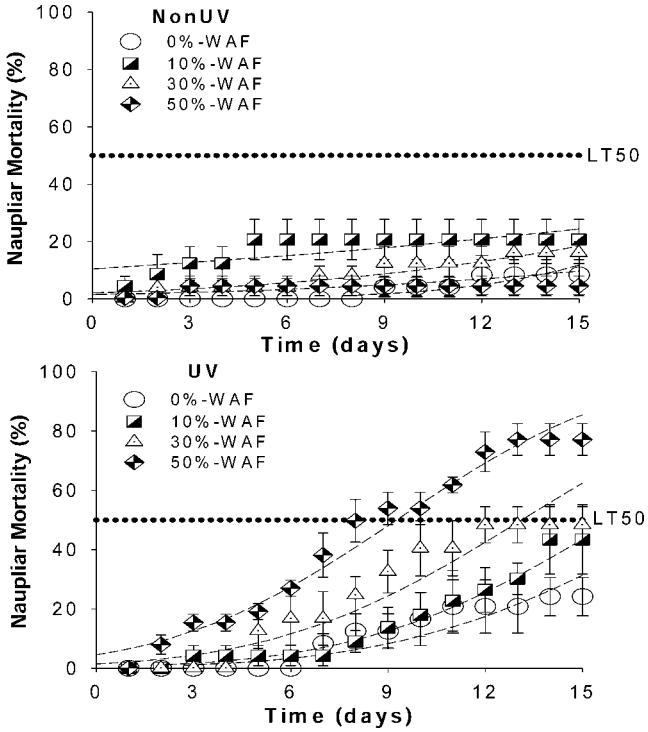

Fig. 4. Naupliar mortality in exposures to National Institute of Standards and Technology crude oil water accommodated fractions (WAF) under ultraviolet (UV) and fluorescent (non-UV) regimes. Treatments included 10, 30, and 50% WAF and seawater control (0%). Long dashed lines represent the predicted probit curves, whereas the dotted line represents the estimated median time to lethality (LT50).

The GLiM analysis of control naupliar-to-copepodite developmental curves estimated 12.5 and 14.0 d for 80% naupliar development into copepodites under fluorescent and UV light regimes, respectively. Within the 30 and 50% WAF treatments, curve analysis based on slopes and intercepts found that the differences in developmental curves between UV and fluorescent light regimes were significant (Fig. 5). Nauplii exposed to 30 and 50% WAF under UV light developed, on average, 41% (i.e., 2 d later) and 63% (i.e., 4 d later) slower, respectively, than nauplii under the same WAF but fluorescent light conditions. Development times across all treatments under fluorescent lighting were similar to those seen from the WAF full life-cycle exposures described earlier.

Summarized WAF crude oil effects on A. tenuiremis

Table 2 presents a summary of all the endpoints for which favorable or adverse effects, relative to controls, were observed for copepod exposures to NIST crude oil WAF. Overall, naupliar survival as well as nauplius-to-copepodite and copepod-

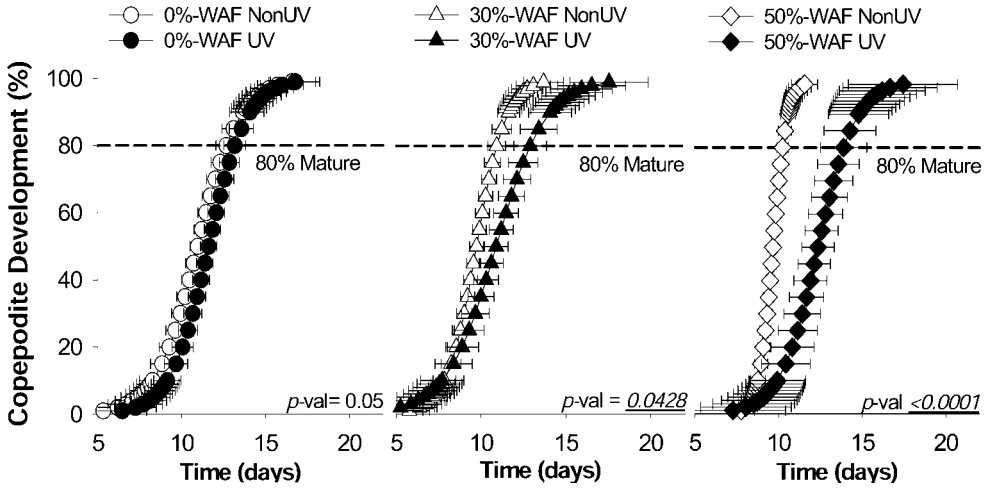

Fig. 5. Estimated nauplius-to-stage-1 copepodite development curves of *Amphiascus tenuiremis* chronically exposed to National Institute of Standards and Technology crude oil water-accommodated fraction (WAF) serial dilutions under ultraviolet (UV) and fluorescent (non-UV) regimes. The *p* values represent within-treatment regression differences based on slopes and intercepts. Curves for non-UV and 10%-WAF are not shown.

ite-to-adult development times were adversely affected in exposures to 100% WAF. Correspondingly, 100% WAF also resulted in a longer life-cycle duration and a reduced projected population growth with time. The presence of UV light generally exacerbated negative effects (i.e., survival and development) attributable to WAF.

Seawater NIST WAF chemistry analysis

Percentage recoveries of the surrogate internal standards, 2-fluorobiphenyl and *p*-terphenyl-d_{14}, were 90% ± 3% and 143% ± 4%, respectively. Nearly 50% of the total PAHs in the NIST 100% WAF were composed of low-molecular-weight naphthalenes (two rings), followed by the larger phenanthrene (28%) and fluoranthene (11%) (Table 3). The NIST crude oil PAHs benzo[*a*]anthracene and benzo[*a*]pyrene were not detected in our WAF. Seawater controls had nondetectable levels of PAHs for all samples.

DISCUSSION

Coastal environments are the shoreline ecosystems most vulnerable to crude oil contamination [25]. Oil spills near these areas threaten benthic fauna and flora, because oil may serve as a source of WAF to surface and pore water. Among benthic fauna, meiobenthic crustaceans are particularly sensitive to oil contamination and often are the first community to show toxic effects (e.g., density declines of harpacticoid copepods; see, e.g., [11,12]). Even short exposures to crude oil and to crude oil WAF can compromise copepod survival and population maintenance [12,26]. For example, Carman and Todaro [12] found that after 7 d of exposure, male and juvenile copepodite densities of two of the four most common Louisiana salt marsh copepod species (*Pseudostenhelia wellsi* and *Coullana* sp.) were reduced by PAH-contaminated sediments from a nearby offshore petroleum production site. A 6-d exposure to 200 ppb of crude oil WAF caused 50% mortality in the coarse-grained sediment–dwelling copepod *Halectinosoma curticorne* [26].

In the past, few monitoring/testing tools have been available

Table 2. Summary of the survival, developmental, and reproductive effects of National Institute of Standards and Technology crude oil water-accommodated fraction (WAF) on *Amphiascus tenuiremis*[a]

Bioassay endpoints	WAF effects observed
N survival	100% (−)
Development from N-to-C	10% (+), 50% (−), 100% (−)
C survival	None
Development from C-to-F	100% (−)
Development from C-to-M	30% (−), 100% (−)
Sex ratio	None
Fertilization failure	None
Brood size	None
Hatching success	100% (−)
Estimated population size	10% (+), 30% (+), 50% (+), 100% (−)
Total life-cycle time	10% (+), 30% (+), 100% (−)
UV-mediated mortality	50% (−)
UV-mediated development	30% (−), 50% (−)

[a] Plus and minus symbols represent positive and negative effects, respectively, relative to controls ($p < 0.05$). C = copepodite; F = adult female; N = nauplius; UV = ultraviolet light.

Table 3. Polycyclic aromatic hydrocarbon (PAH) analysis of freshly extracted, 100% National Institute of Standards and Technology crude oil water-accommodated fraction (WAF) using U.S. Environmental Protection Agency SW-846 Method 3510C[a]

Analytes	PAH concentration in 100% WAF (μg/L)	Instrument detection limits (μg/L)
Acenapthene	8.3 ± 0.37	≥0.17
Acenaphthylene	2.97 ± 0.15	≥0.16
Anthracene	ND[b]	≥0.26
Benzo[*a*]anthracene	ND	≥0.44
Benzo[*b*]fluoranthene	ND	≥0.12
Benzo[*k*]fluoranthene	ND	≥0.27
Benzo[*a*]pyrene	ND	≥0.12
Benzo[*ghi*]perylene	ND	≥0.09
Chrysene	ND	≥0.18
Dibenzo[*a,h*]anthracene	ND	≥0.09
Fluoranthene	14.15 ± 2.49	≥0.16
Fluorene	ND	≥0.19
Ideno[1,2,3-*cd*]pyrene	ND	≥0.14
Naphthalene	67.09 ± 3.79	≥0.24
Phenanthrene	37.2 ± 1.89	≥0.17
Pyrene	4.46 ± 1.73	≥0.16
Total PAHs	134.19 ± 4.37	

[a] (http://www.epa.gov/epaoswer/hazwaste/test/pdfs/3510c.pdf; $n = 3$ replicates/sample).

[b] ND = not detected or below the instrument detection limit for the individual PAH.

to robustly determine threshold WAF concentrations below which toxicity is not a 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 life cycles. One frequently overlooked but ecologically important lower-trophic-level community of high potential value in this regard is meiobenthic copepods. In the present study, we expanded the utility of copepod-based, full life-cycle testing in microplates (ASTM E2317-04 [14]) to be able to measure individual- to population-level safe WAF crude oil concentrations as well as reference copepod responses to the well-characterized NIST petroleum crude oil standard reference material. This bioassay is comprehensive but has simple logistics and reduced requirements for test water and wastewater (<2 L for all tests presented here).

In the present study, the highest WAF produced from the NIST crude oil standard (100% WAF; total PAHs, 134.2 ± 4.4 μg/L) was comparatively low in total PAHs relative to other crude oil types [2,9,10], but it still enhanced naupliar mortality by 27% and significantly reduced naupliar and copepodite development into the next life stage. Delayed development into males was seen across all WAFs, but only the 30 and 100% WAFs were significantly delayed relative to controls. Interestingly, nauplius development into the copepodite stage was enhanced by a mild, 10% WAF exposure, suggesting a WAF conditioning or hormesis effect. On the negative side, petroleum hydrocarbons (e.g., PAHs) can cause direct narcosis and inhibit ecdysis in crustaceans [27,28], which in turn can result in mortality and alteration of processes such as molting, development and reproduction. Naphthalenes, which were the major constituents in our NIST WAF (50% of total PAHs), likely were the most important contributors to the naupliar toxicity and developmental effects observed here. With regard to UV-enhanced acute and chronic toxicity, the higher-molecular-weight PAHs fluoranthene, phenanthrene, and pyrene (Table 1) were at sufficiently high concentrations in NIST WAF to have contributed to phototoxicity in these relatively UV-transparent microcrustaceans [29–31]. The observed slower rates of development into male versus female adults is not unique to NIST WAF; *A. tenuiremis* males generally are more sensitive than females to acute and chronic pesticide and PCB exposure [17,32,33], possibly resulting from sex-specific differences in body lipid concentrations [17] and/or higher surface to volume ratios in the smaller male morphs. In contrast, an enhancement in naupliar-to-copepodite development at 10% NIST WAF could potentially be explained by a positive induction of cytochrome P450–dependent xenobiotic monooxygenase isozymes. Studies [34–36] have suggested that induction of cytochrome P450 isozymes by relatively low levels of PAHs can result in an acceleration of the molt cycle (i.e., development) by increasing titers of the molt-regulating hormone 20-hydroxyecdysone. For example, Snyder [36] 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 ecdysteroid metabolism, molting, and development.

In the present study, none of the NIST WAFs tested had significant effects on mean fertility or offspring production. Embryo hatching success was the only reproductive endpoint reduced at 100% WAF, and this likely resulted from embryo narcosis. Multigeneration projections of population growth via Leslie stage-matrix models predict elevated naupliar production at 10, 30, and 50% NIST WAFs but reduced production at 100 WAF. Projected naupliar production was influenced primarily by differences in overall copepod development (i.e., 3- and 1-d accelerations in the 10 and 30% WAFs, but 3.5-d decelerations in the 100% WAF) and by differences in stage-specific survival rates (e.g., at 100% WAF), embryo hatching success, and viable offspring production totals per individual (reproductive success). The population growth model presented here was (should be) used only as a tool to predict best-case potential relative population-level outcomes in the presence of toxicants, in the absence of predation and disease, and with abundant food. We do not mean to imply that low levels of WAF will provide an ecological advantage to exposed copepod populations, just that minimal deleterious population-level effects of NIST WAF at less than full-strength levels could be found in the somewhat unnatural non-UV state. In fact, in the present study, a more rapid turnover of reproductive life-cycles at lower WAF concentrations did not result in higher viable production rates, as might be expected, for example, by a hormetic or reproductive stimulant.

We also evaluated the effect of light regime (UV vs cool fluorescent) on WAF (10, 30, and 50%) acute and chronic toxicity. Relatively short exposures (<16 d) to fairly low WAF concentrations (i.e., 30 and 50% WAF) in combination with low UV levels (i.e., fourfold lower than sunlight UV levels) were sufficient to cause negative impacts on naupliar survival and development. Even though UV exposure alone increased control naupliar mortality by 15% relative to non-UV controls, the UV-WAF combination resulted in strongly enhanced naupliar mortality, particularly in the 50% WAF treatment. Furthermore, nauplii exposed to 30 and 50% WAFs in the presence of UV light showed significant delays in development into the copepodite stage. These results were likely related to UV-induced photoactivation of crude oil WAF [37]. Numerous studies have described the photoactivation of certain PAHs (anthracene, benzo[*a*]pyrene, fluoranthene, pyrene, benzo[*a*]anthracene, and dibenzothiophene) by UV light [29–31,37]. The PAH phototoxicity results from energy absorption by PAH molecules leading to super-reactive, highly oxidizing free radicals that can damage biological macromolecules, such as DNA [30]. In WAF exposures from various crude oil sources and under UV conditions, Pelletier et al. [31] found several-fold increases in sublethal toxicities to embryos of the bivalve *Mulinia lateralis* compared to WAF toxicities under cool fluorescent light.

Fluoranthene was the only strongly photoactive PAH detected in our 100% WAF. However, we do not know if this PAH at a concentration of 14.2 ± 2.5 μg/L could be solely responsible for the observed UV-mediated acute and subacute toxicity. The phototoxic PAH dibenzothiophene [37] is one of the major PAHs found in the NIST crude oil standard at a concentration of 33 ± 2 μg/g. This PAH, however, was inadvertently excluded from our WAF chemical analysis because of a missing internal reference standard, so we are unsure of its concentration in our NIST WAF. Perhaps, the combined phototoxicity of fluoranthene and dibenzothiophene could have been responsible for the effects observed here.

In summary, high NIST WAF concentrations likely pose an acute and chronic risk to the early life stages of *A. tenuiremis* and other copepods. These risks could potentially be exacerbated by UV irradiation, because these organisms often live at the highest densities exposed to solar UV light on the surface of intertidal mudflats. Also, low WAF concentrations

may accelerate development with no negative effects. Copepods comprise 10 to 40% of the meiobenthic fauna [38] and constitute an important component of the benthic community in coastal and estuarine areas [11,38]. Effects of crude oil WAF on meiobenthic copepods, such as reduced naupliar survival and offspring production, could reduce the food available to higher trophic levels [39]. Conversely, stimulation of copepod production by low WAF concentrations could enhance their trophic value to higher consumers. Further studies of benefit to oil spill management should focus on additional WAF crude oil types/sources in conjunction with NIST WAF as a common positive reference standard. The demographic modeling capabilities of this bioassay should prove useful to petroleum ecological risk assessment and prediction and, especially, for postspill remediation efforts focused on answering the difficult question of ''how clean is clean enough?''

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