Utility of Meiobenthos for Risk Assessment of Crude Oil WAFs: Rapid Copepod-based Approaches for Evaluating Reproductive and Population-level Toxicity

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Context

- Risk assessments of oil-spill effects on wildlife require *population-level* information to have highest predictive power… i.e., require fitness-relevant endpoints such as mating success and offspring production and survival.

- Despite this need, *rapid* full lifecycle bioassays have been unavailable for sediment infauna most at risk of oil exposure. Fortunately, meiobenthos are useful in this regard… with some species amenable to both water AND sediment-based testing…

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Objectives

So, this morning I will discuss recent work in my lab focused on developing a meiobenthos-based lifecycle assay for crude oil WAFs -- benchmarked against the NIST crude oil standard -- using our ASTM-standard harpacticoid copepod bioassay, and UV, non-UV exposures.

Designation: E 2317 – 04

Standard Guide for
Conducting Renewal Microplate-Based Life-Cycle Toxicity Tests with a Marine Meiobenthic Copepod[1]

This standard is issued under the fixed designation E 2317; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

- I will show you how harpacticoid culture can be used to assess fitness-relevant endpoints (e.g., survival, development rates, sex ratio shifts, fertilization success, egg quality, clutch size, offspring production) useful for modeling WAF population effects with simple life-table based approaches…

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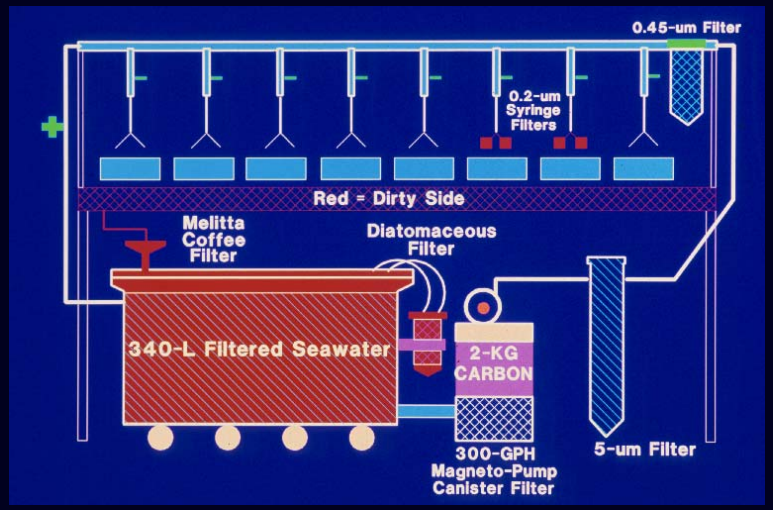
Methods for microplate bioassays with NIST & *South Louisiana Crude*…

Generation of WAFs for testing…

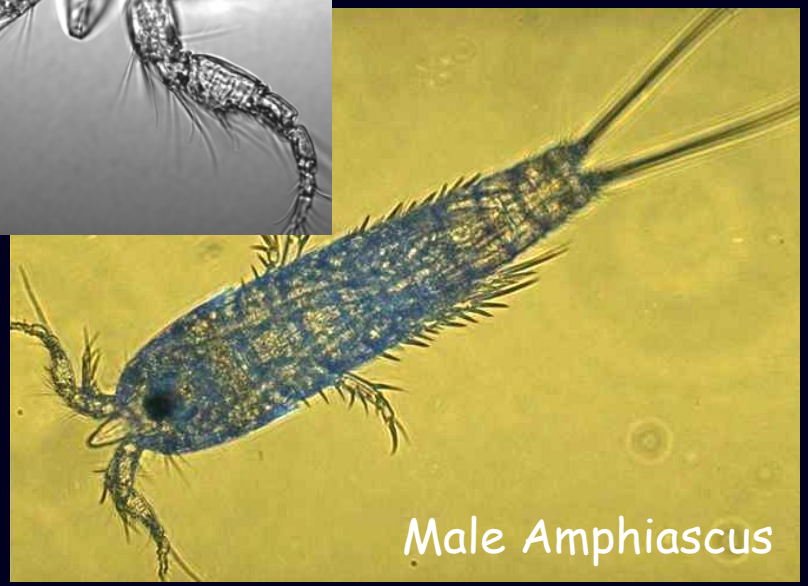
Assessment of survival, development rates, reproductive success, Leslie matrix pop. growth models,…

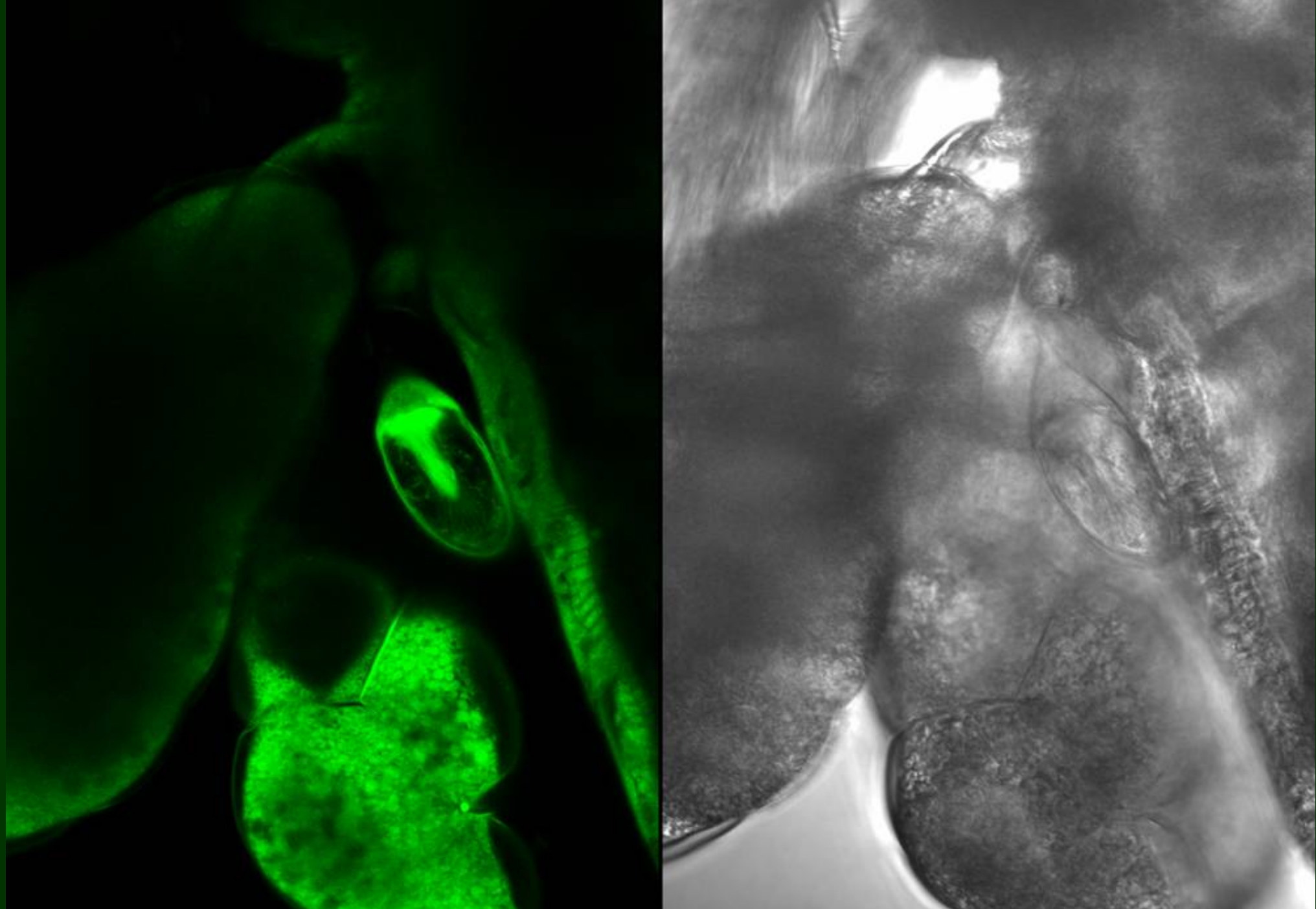
Copepods for testing are stock cultured in muddy sediments…



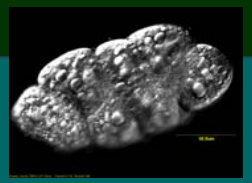
Amphiascus tenuiremis

Our pet copepod shows strong sexual dimorphism… males are skinny w/ swollen antennae for clasping





Discrete Lifestages of the meiobenthic copepod *Amphiascus tenuiremis* at 25C in 96-well microplate culture (15-35S)



Embryo

2 days hatch

Nauplius (6 stages)

8 days

Copepodite (5 stages)

7 days

Adult Male or Female

1 day mate

Adult Gravid

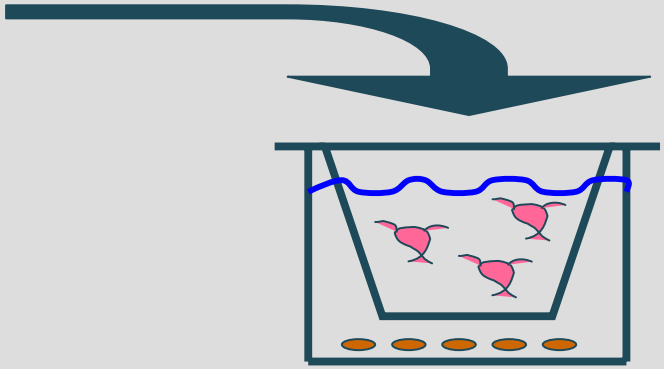
fecundity

- Lifecycle = 18-19 days Egg to Egg
- Avg. Life Expectancy = 47 ± 2 days
- Avg. Clutch = 6.2 ± 2 eggs
- 8-9 Clutches/Life

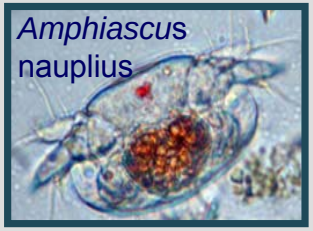
Note: Sediment lifecycle is ~20% faster.

Discrete lifestages make the microplate bioassay work...

~ 200 gravid *A. tenuiremis*
(from lab stock mud cultures)

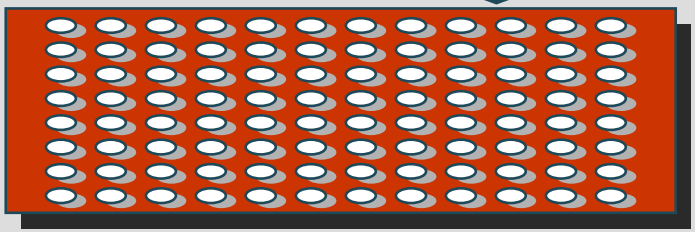


12-well plate with 75 μm meshed-well inserts;
Yields ~ 500 nauplii in < 24 hour



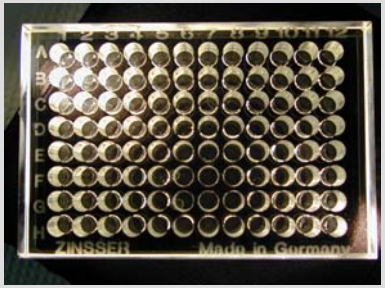
(n ≥ 40 per microplate with 3 plates/trtmnt)

Amphiascus nauplius



96-well Costar® glass-lined or ultra-low attachment polyacrylamide microplate; 200 μl WAF solution per 10 wells every other row.

X-MATINGS



Life-cycle Endpoints:
NEXT SLIDE……..

Possible Copepod Microplate Endpoints

Mortality by Life Stage

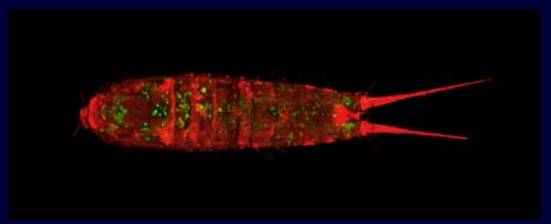
Reproductive Endpoints

- Fertilization Success by Sex
- Abortion rates
- Egg Quality & Hatching
- Clutch Size
- Time to 1st and 2nd clutch
- F_0 Production Rates through 2 clutches ($F_{1,2}$)
- Hormonal/endocrine change (male lipovitellin, ecdysone @ virgin-adult)
- Population growth parameters: λ, r, stable-age structure, projected pop. sizes by treatment.

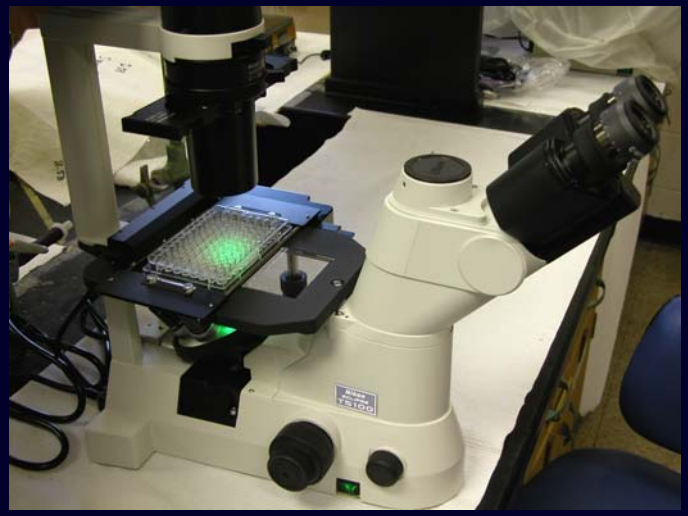
Developmental Endpoints

- Teratogenesis & Necrotic eggs
- Times to Copepodite and Adult
- Body Size and Phenotype
- $F_{0,N}$ Sex Ratios
- Hormonal/endocrine change (stage-specific ecdysone balance, vitellogenesis in males/females)

ENDPOINTS IN 'RED' ARE PROPOSED FOR OECD Copepod Test Guideline



What the microplate test looks like in practice…



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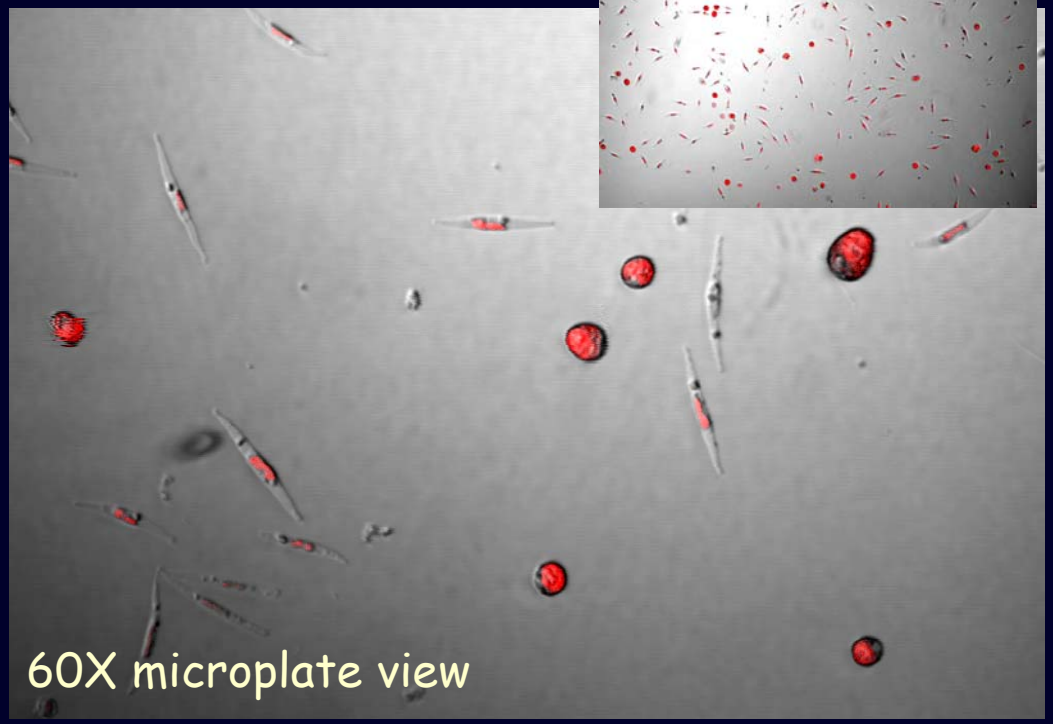
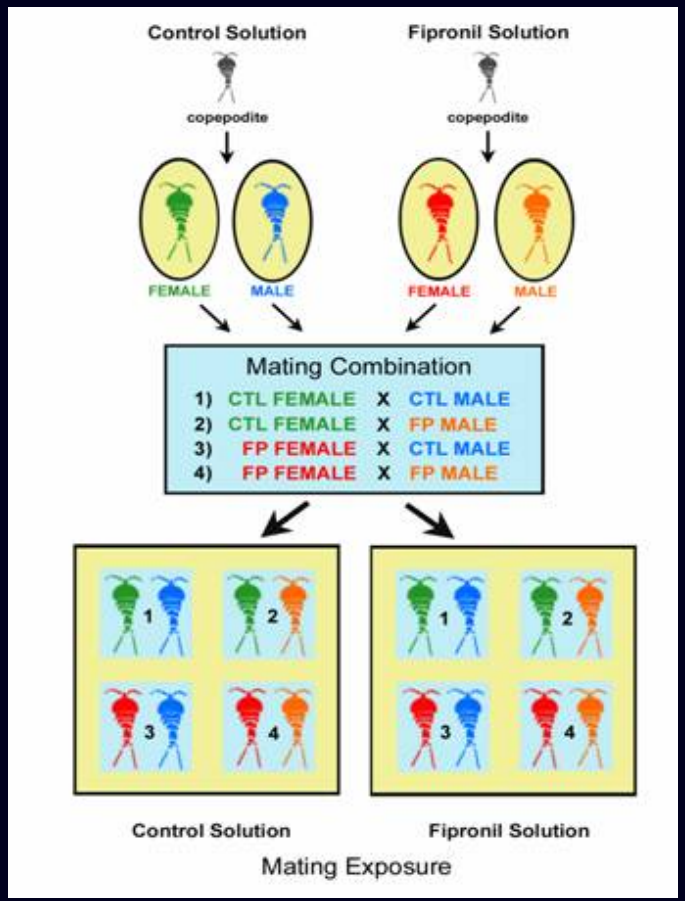


Here's the fun part… detection of sex-linked effects..

Since nauplii are reared singly to adulthood, individual copepod cross-matings can be manipulated between virgin males and females both within and across treatments in "clean" or WAF-dosed seawater. Virgin females are mateable for at least 16 days.

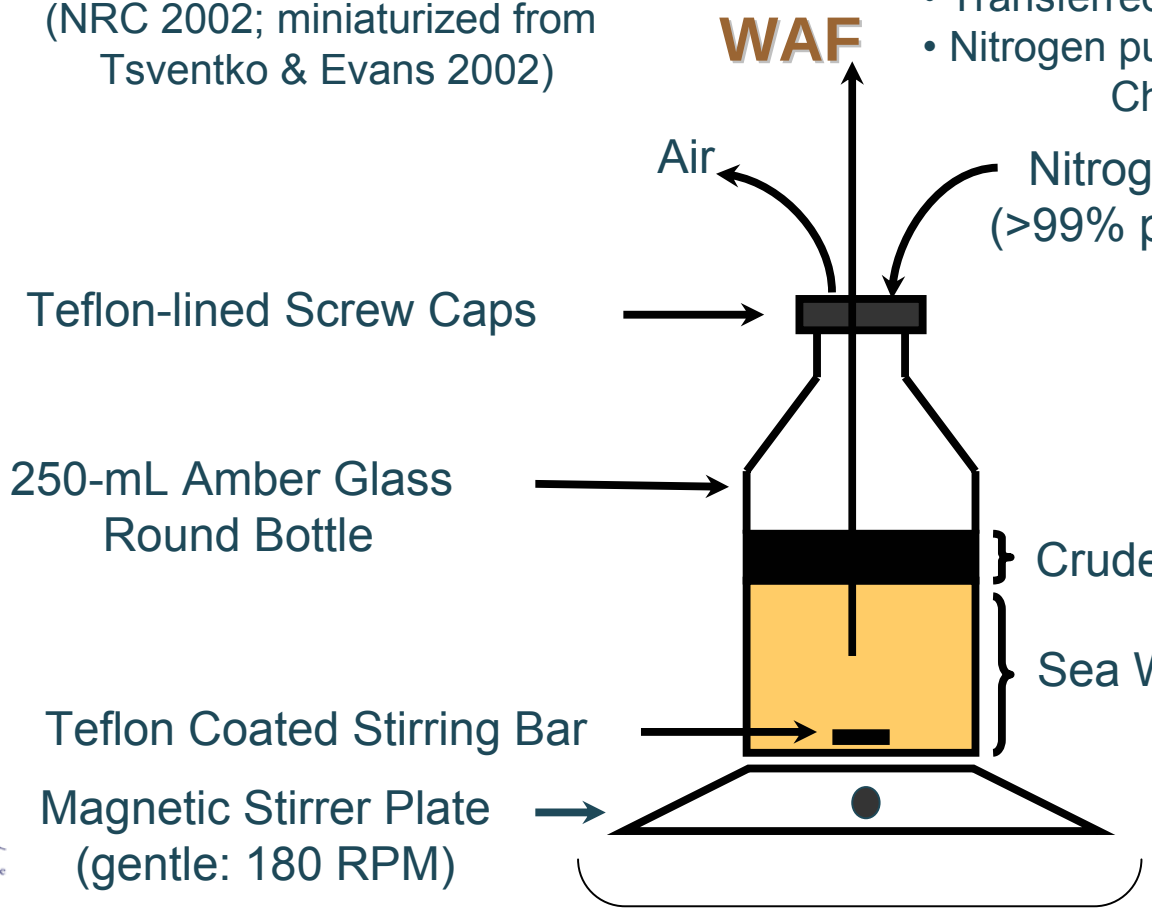


WAF copepod lifecycle bioassay setup…

METHODS FOR WAF GENERATION

(NRC 2002; miniaturized from Tsventko & Evans 2002)

- WAF harvested with stainless-steel needle attached to a 50-mL Hamilton gas-tight syringe.
- Transferred into solvent-cleaned amber bottle.
- Nitrogen purged and stored in the dark @ 4°C; Chemical analysis within 24 h.



- **2-mL NIST or South Louisiana Crude Oil**
- **60-mL 0.22 µm-filtered seawater (30‰)**

System set-up in incubator. Oil:Water stirred in the dark for 36 hours at 20ºC.

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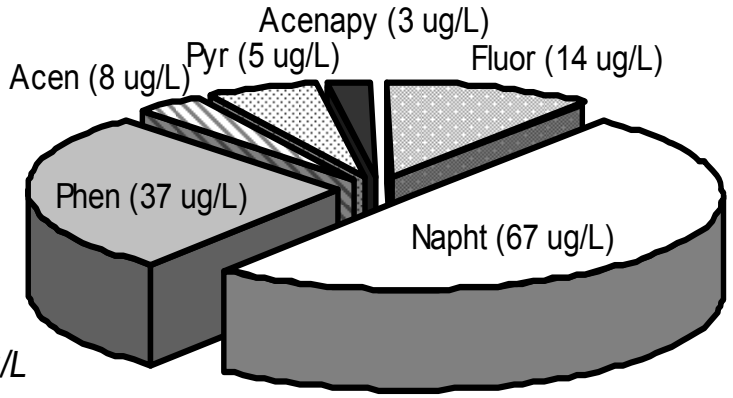
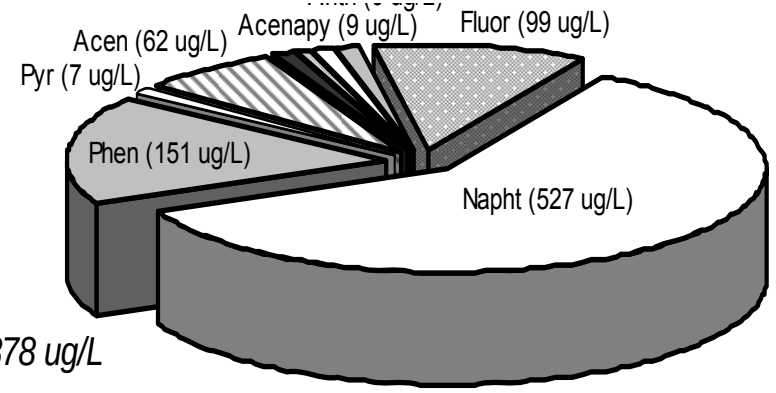
NIST



Total PAHs= 134 ug/L

Louisiana



Total PAHs= 878 ug/L

Analytes:

Acenapthene (Acena)
Acenaphthylene (Acenapy)
Anthracene (Anth)
Benzo [a] anthracene (B[a]anth)
Chrysene (Chry)
Fluoranthene (Fluor)
Naphthalene (Napht)
Phenanthrene (Phen)
Pyrene (Pyr)

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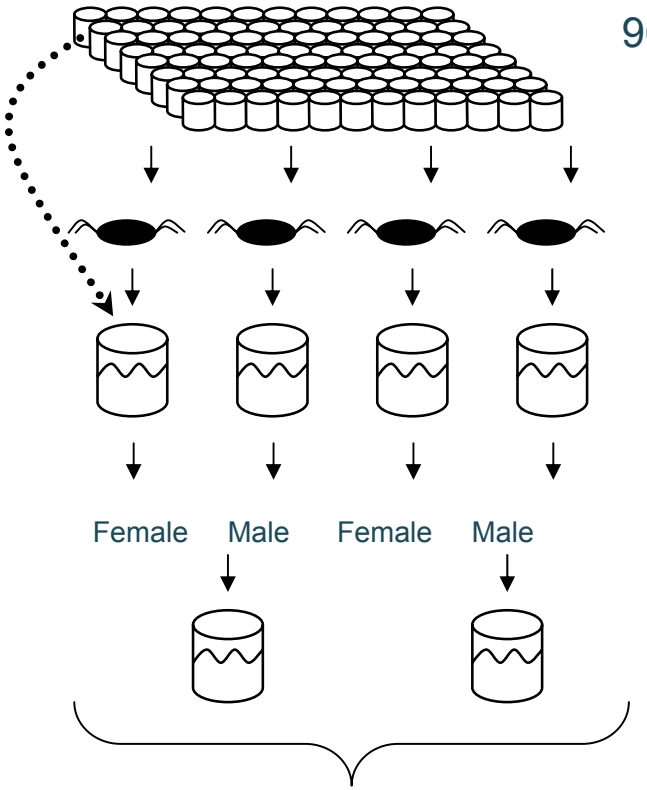
Crude Oil- WAF Microplate Full Lifecycle Bioassay



96- well glass coated microplate

Nauplius (**N**)

Copepodite (**C**)

Adult copepods (**A**)

Mating

n= ≥40 **N** / microplate
3 microplates / WAF conc.
200 µL WAF / microwell

Bioassay Endpoints

N survival, time to **C**-1

C-1 survival, time to **A**dult

Adult sex ratios
Adult reproductive success

Abortion rates
1st and 2nd brood size
Hatching success
Production per female

WAF Exposure treatments
100%, 50%, 30%, 10%, 0% (control)

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Bioassay results…

SURVIVAL OVER 30 DAYS – UV ABSENT

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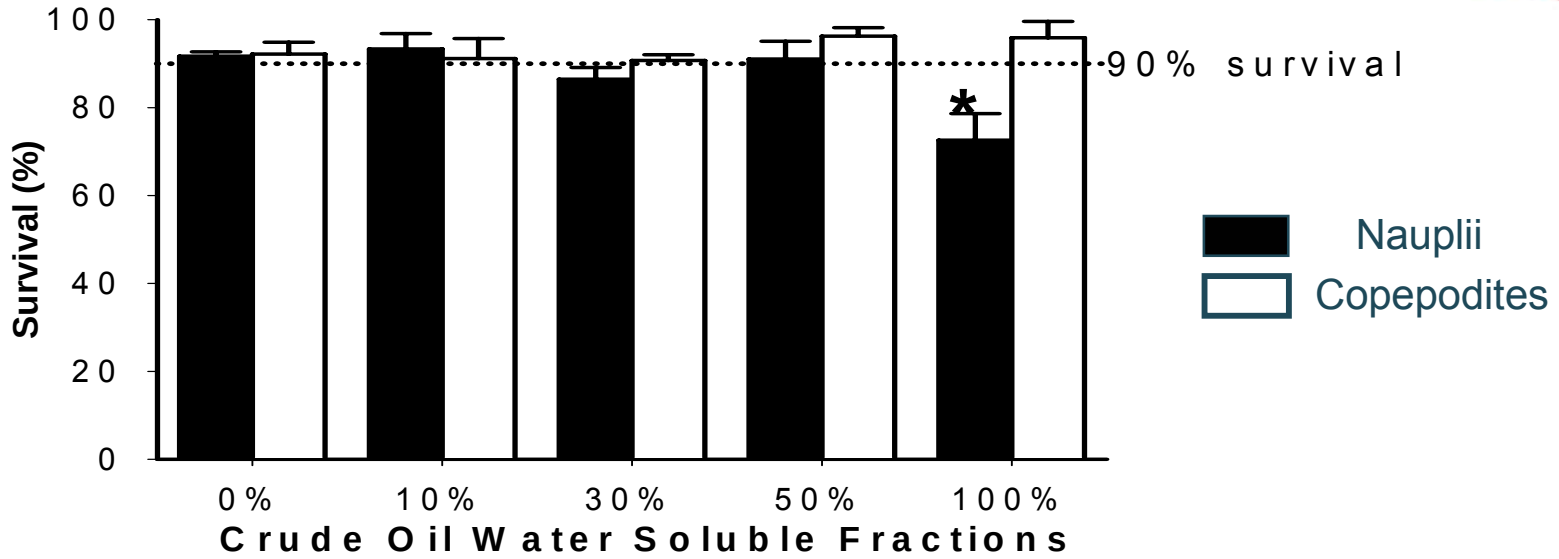

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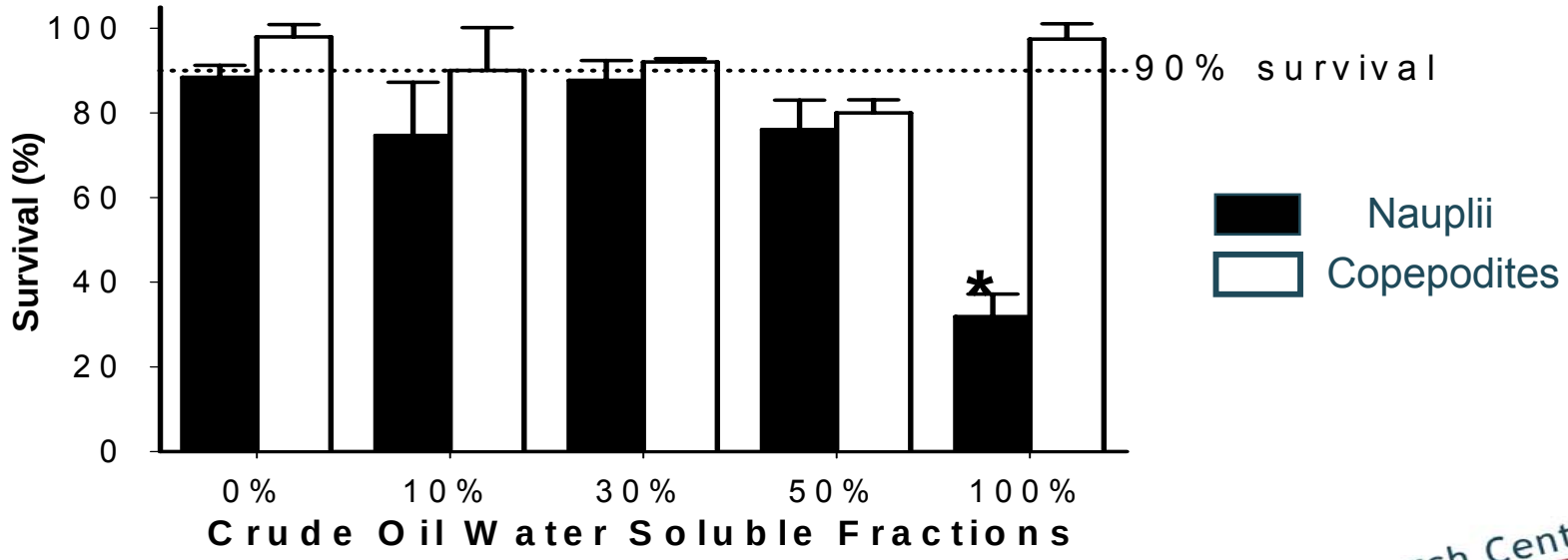

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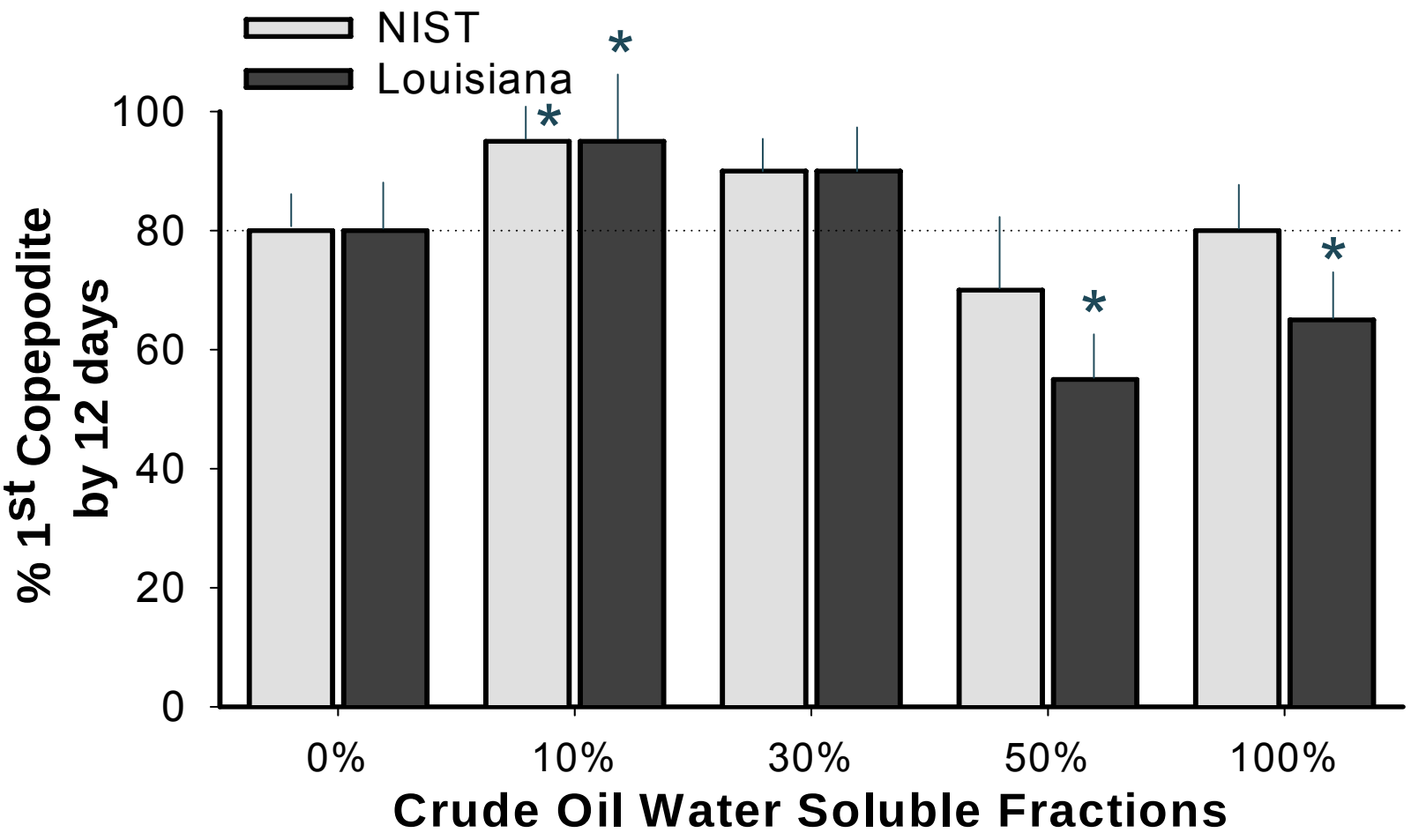
PERCENT OF NAUPLII REACHING FIRST COPEPODITE STAGE BY 12 DAYS OF EXPOSURE



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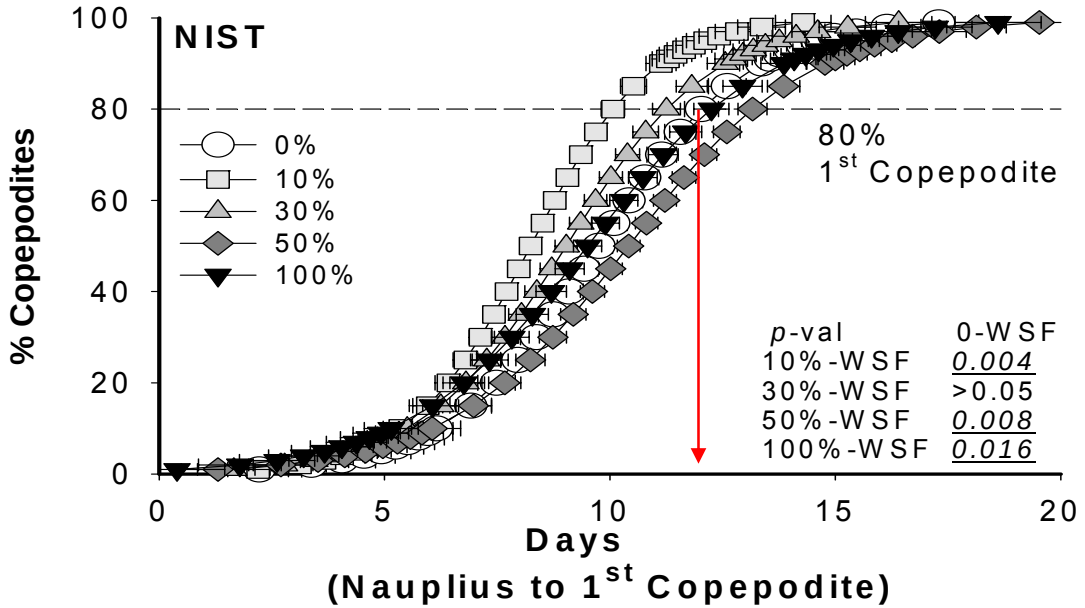
DEVELOPMENT CURVES

NIST



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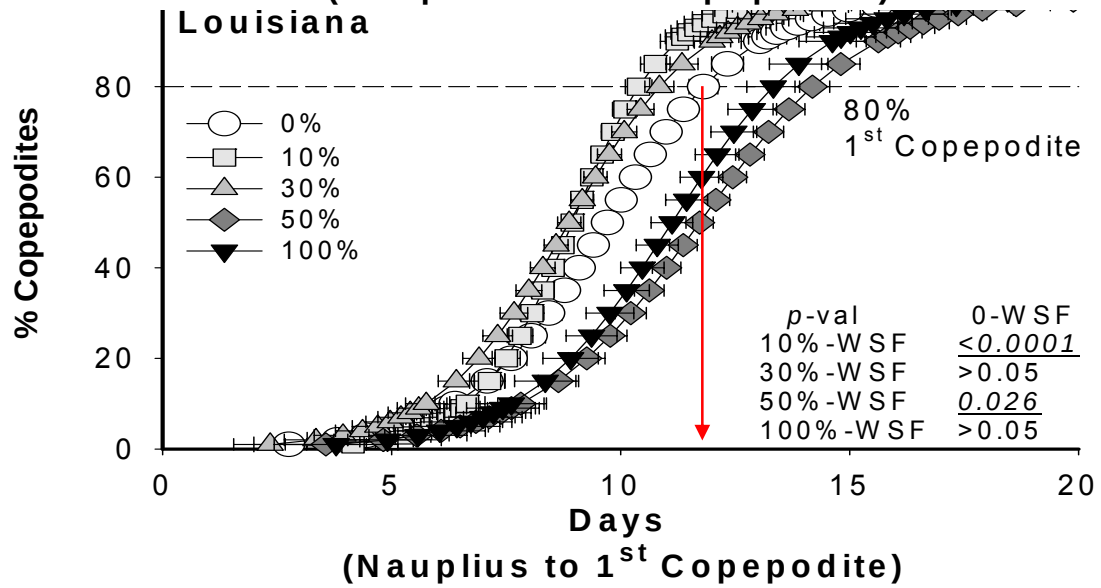

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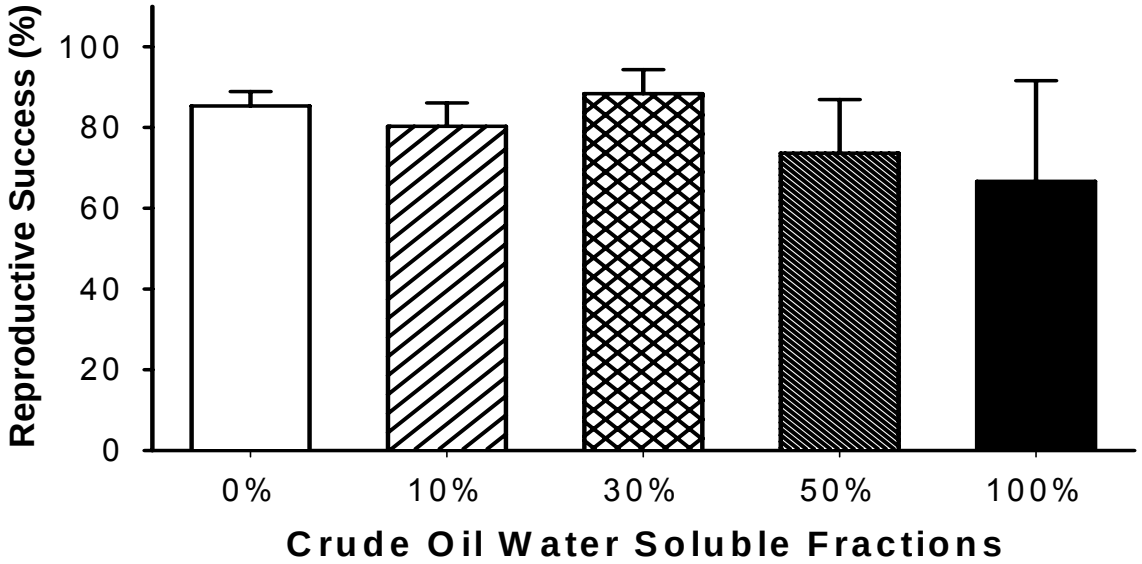
MATING-PAIR REPRO SUCCESS

NIST



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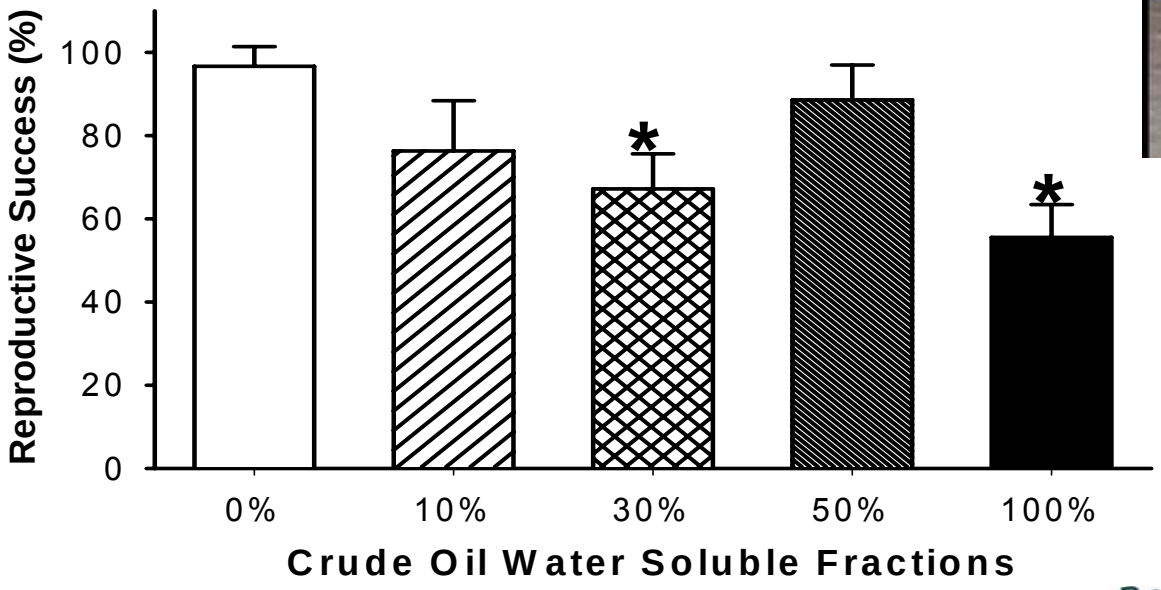

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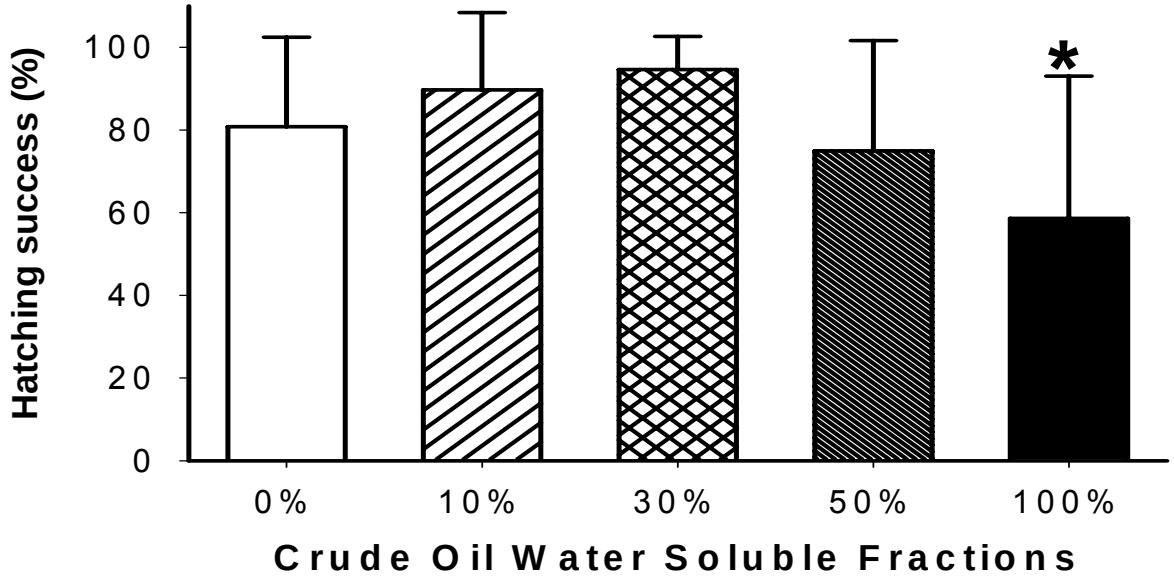
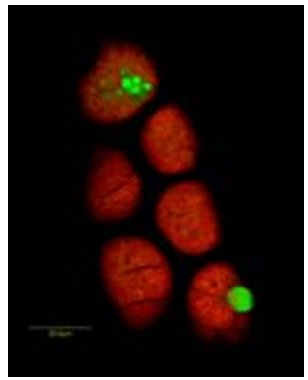
EGG HATCHING SUCCESS

NIST





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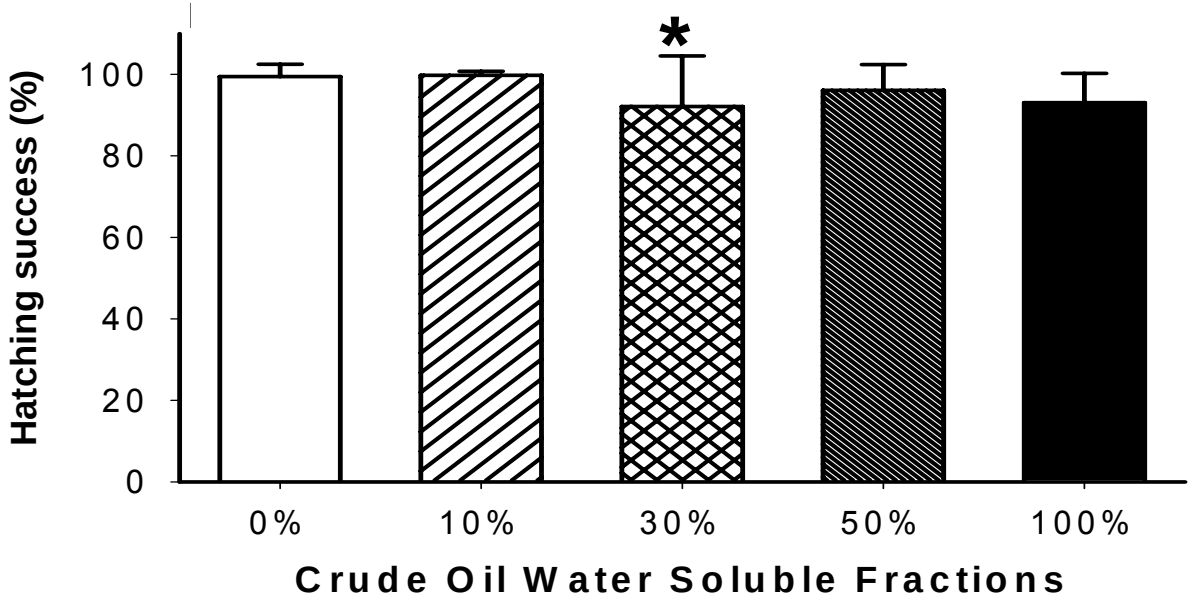

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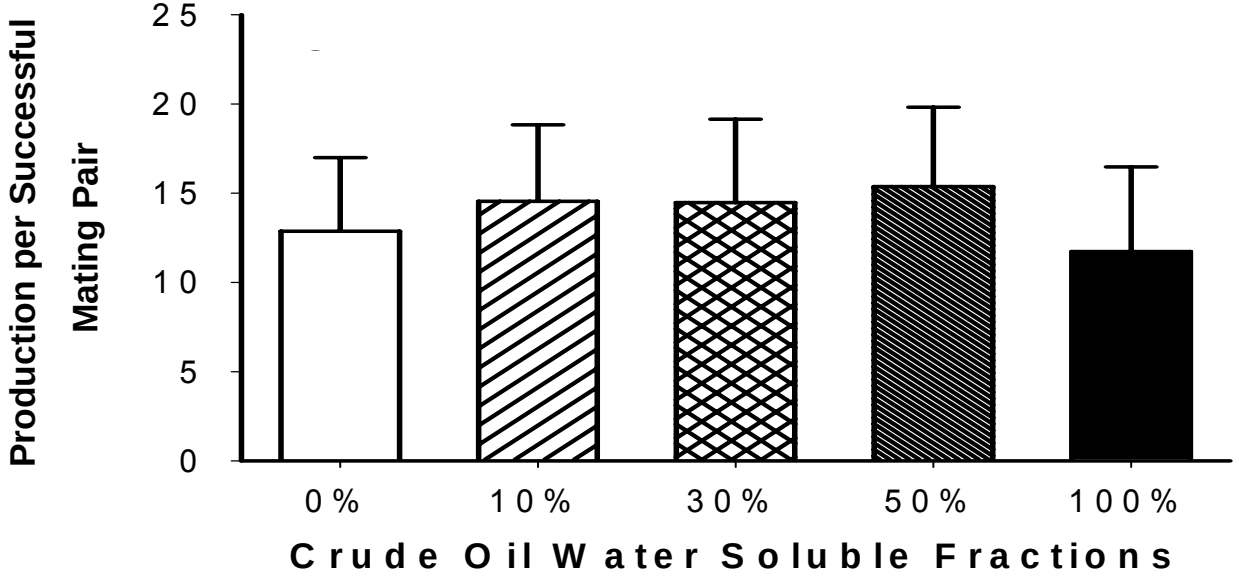
PRODUCTION THRU 2 CLUTCHES: for Successful Pairs

NIST



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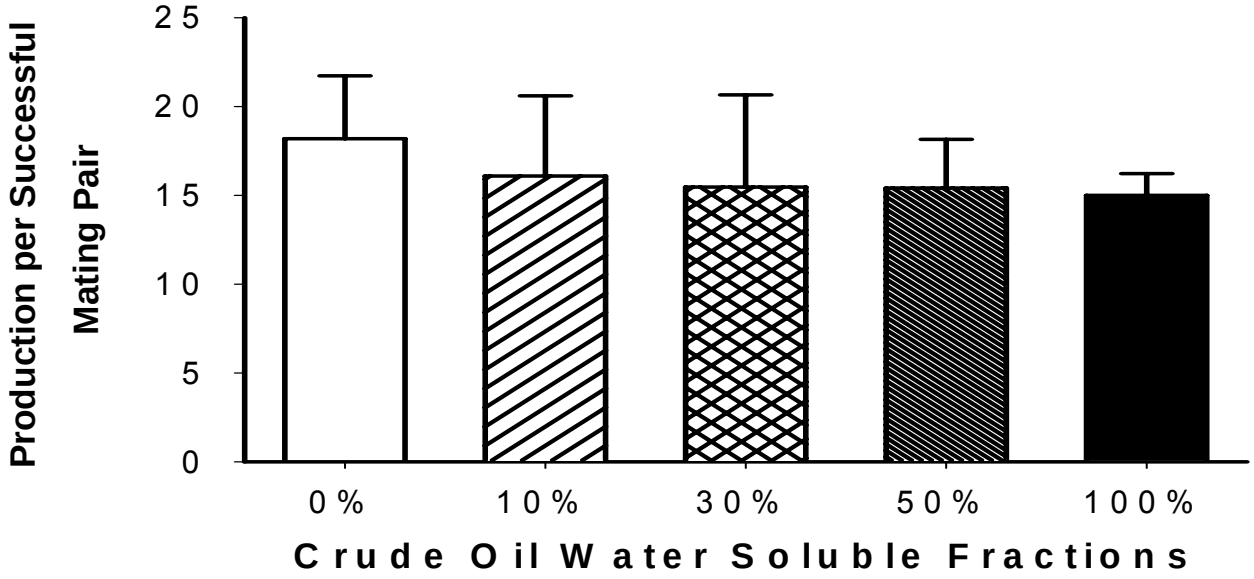

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PRODUCTION THRU 2 CLUTCHES: All pairs

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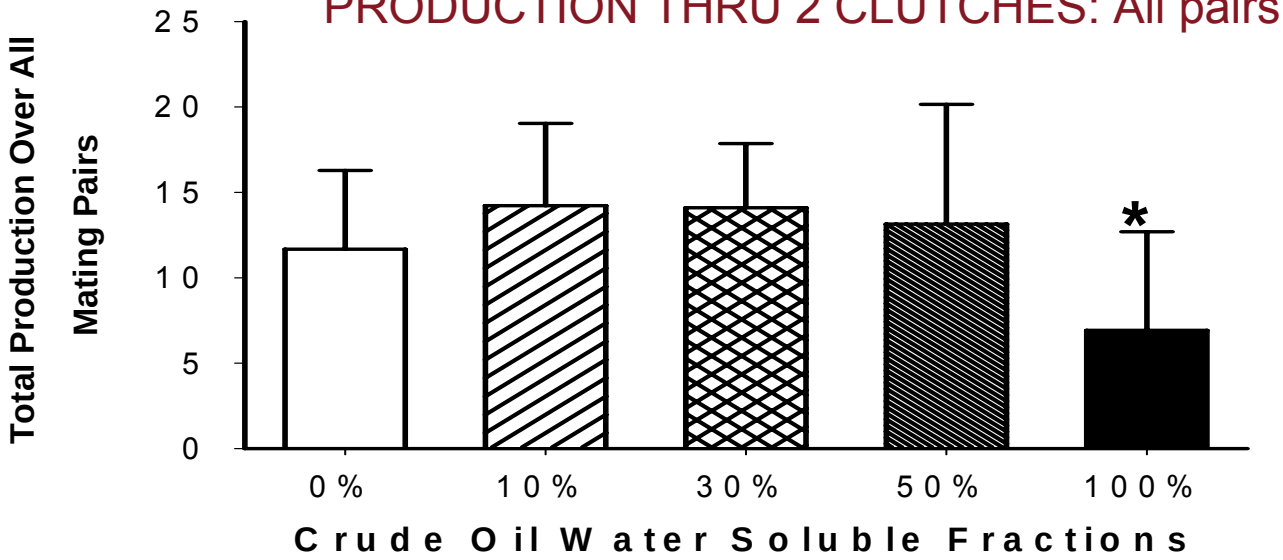

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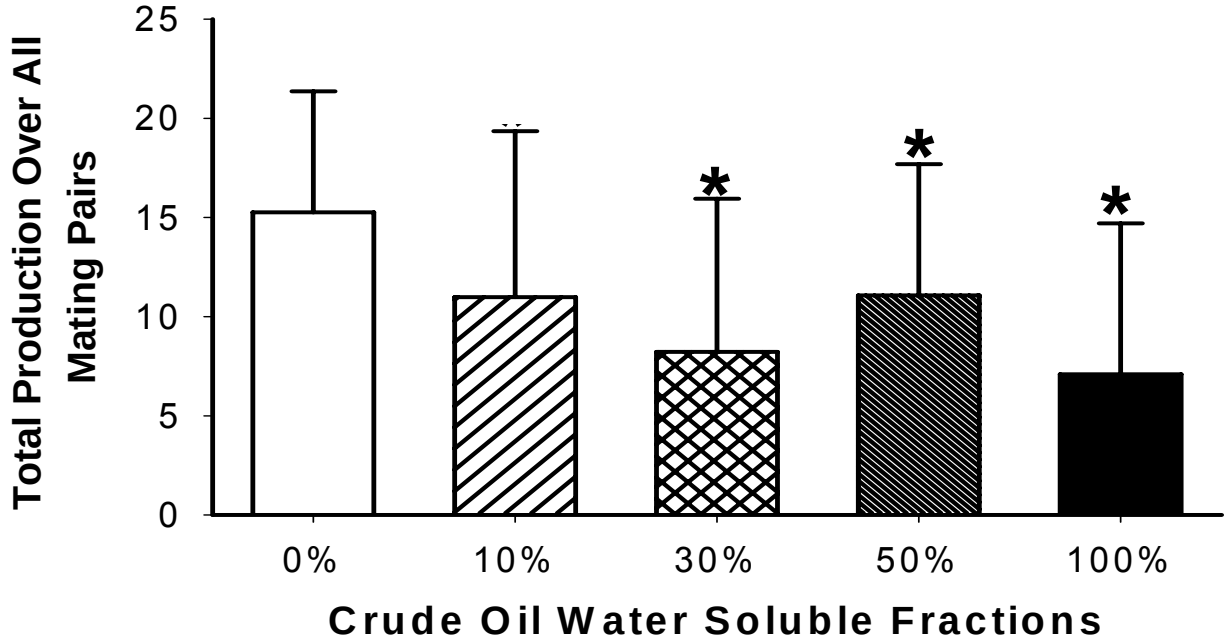

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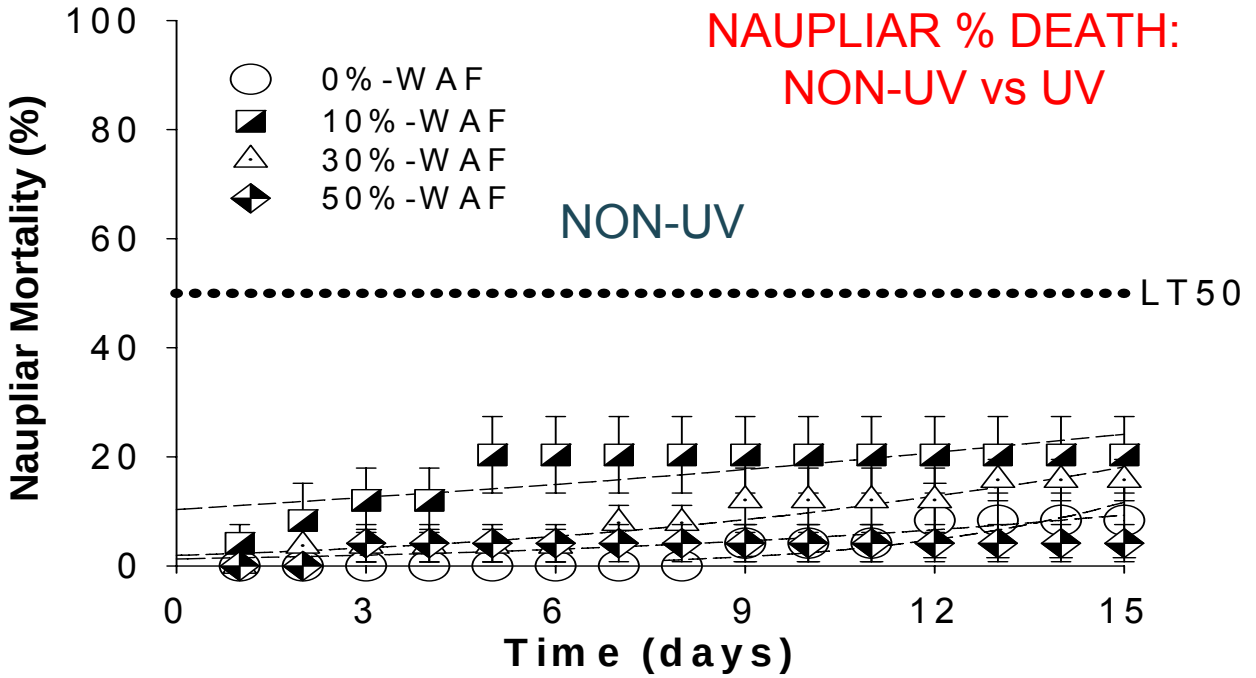
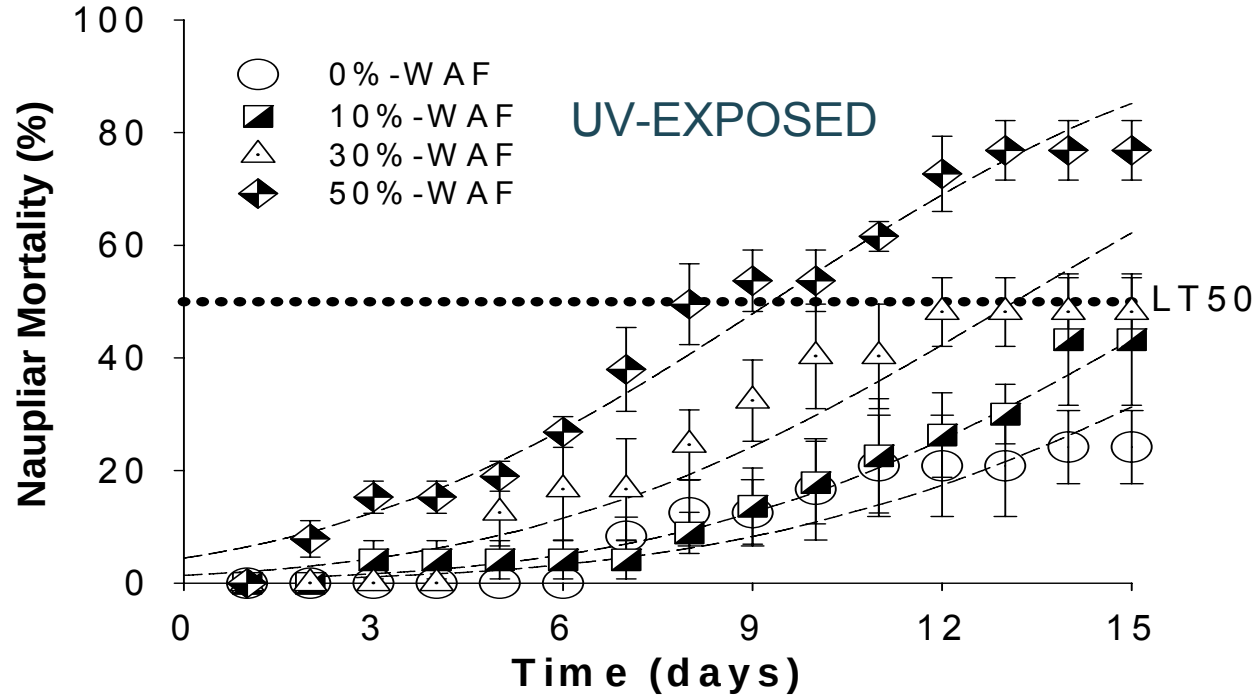
UV enhances PAH toxicity… What about UV exposed microplates… NIST results:

NAUPLIAR % DEATH: NON-UV vs UV





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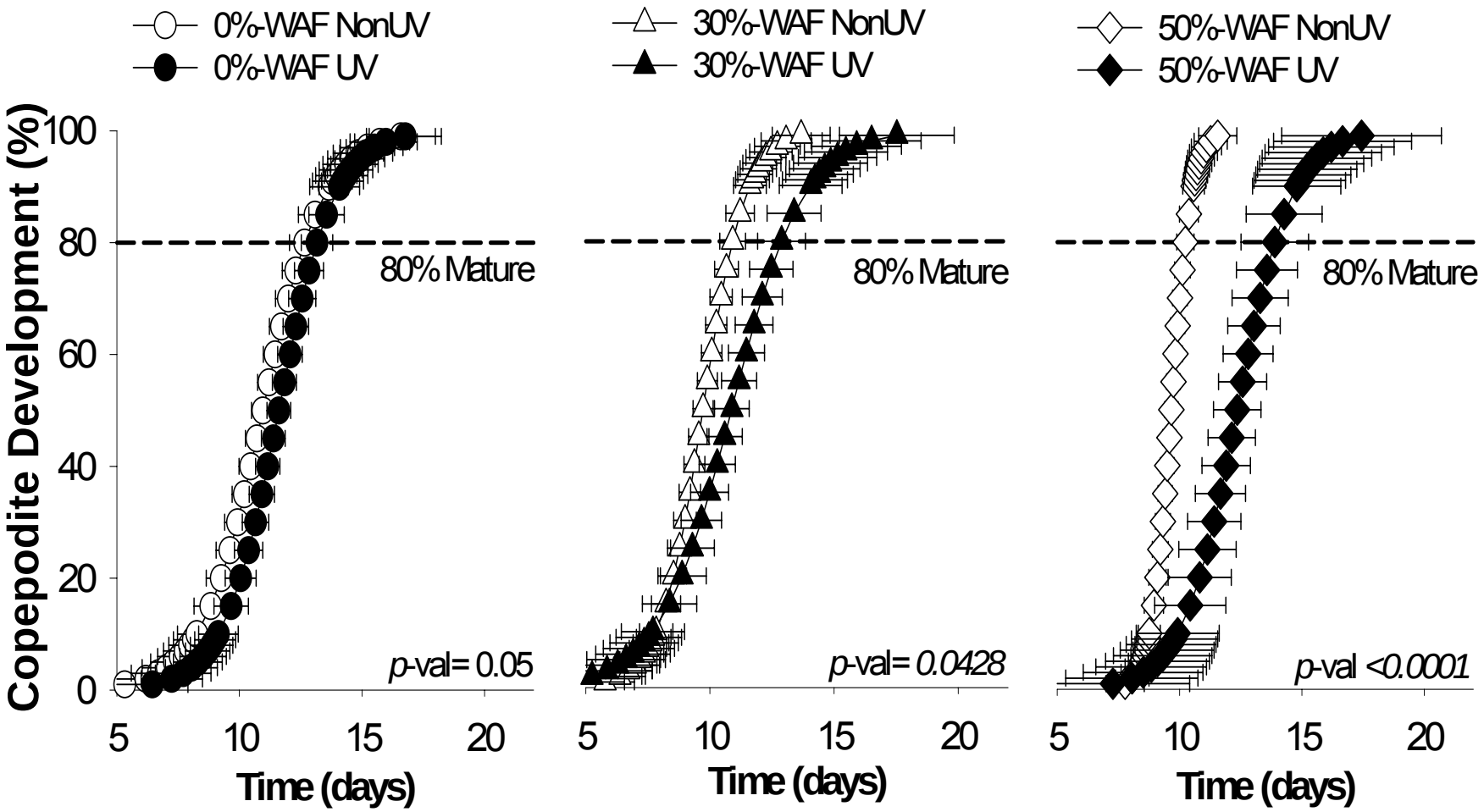

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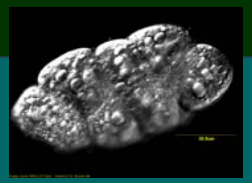
What are the relative risks of WAFs to population growth & maintenance?

Application of NIST & SL Crude Oil WAF results to the Leslie matrix Pop. Growth model…

Discrete Lifestages of the meiobenthic copepod *Amphiascus tenuiremis* at 25C in 96-well microplate culture (15-35S)



Embryo

2 days hatch

Nauplius (6 stages)

8 days

Copepodite (5 stages)

7 days

Adult Male or Female

1 day mate

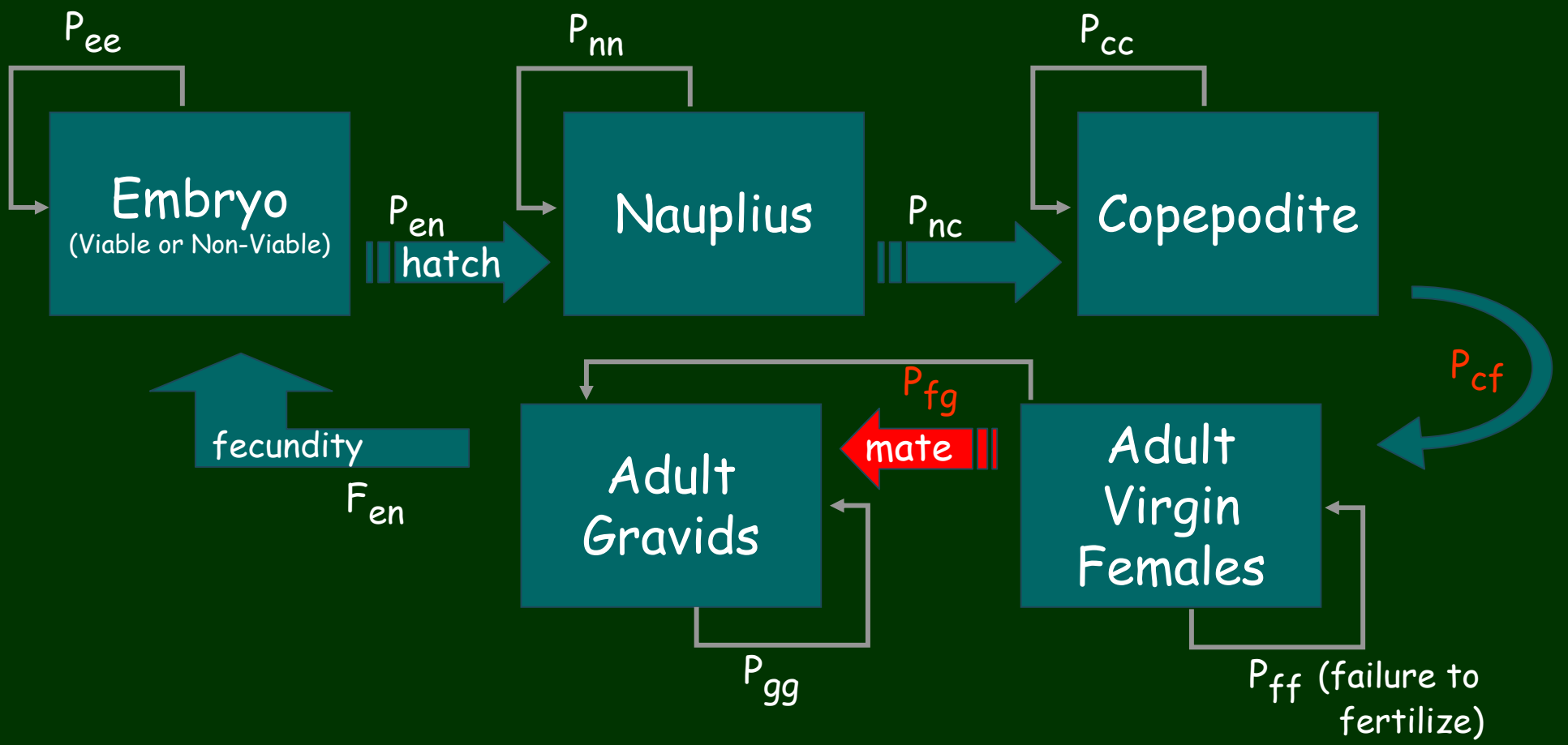
Adult Gravid

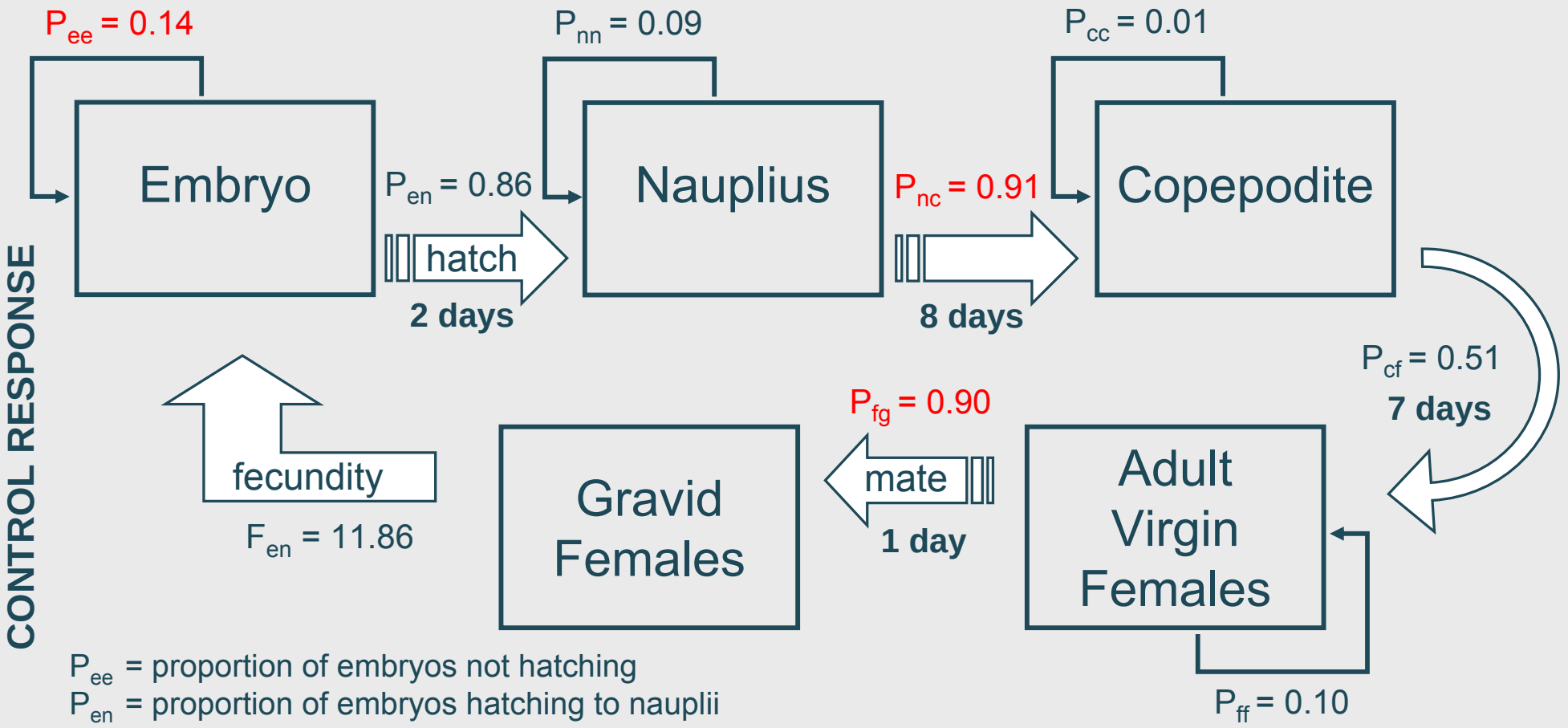
fecundity

- Lifecycle = 18-19 days Egg to Egg
- Avg. Life Expectancy = 47 ± 2 days
- Avg. Clutch = 6.2 ± 2 eggs
- 8-9 Clutches/Life

Note: Sediment lifecycle is ~20% faster.

Matriarchal Leslie-Matrix Stage-Structured Model for Impacts of Developmental and Reproductive Change in *Amphiascus tenuiremis*





P_{ee} = proportion of embryos not hatching
P_{en} = proportion of embryos hatching to nauplii
P_{nc} = proportion of nauplii becoming copepodites
P_{nn} = proportion of nauplii not developing to copepodites
P_{cf} = proportion of copepodites becoming virgin females
P_{cc} = proportion of copepodites not developing to adult
P_{fg} = proportion of mated females becoming gravid
P_{ff} = proportion of mated females remaining barren
F_{en} = fecundity through two broods of nauplii

- Lifecycle = 18-19 days egg to egg
- Avg. Lifespan = 47 $\pm$ 2 days
- Avg. Clutch = 6.2 $\pm$ 3.6 eggs
- 8-9 Clutches/lifetime

Population-level effects: 5-stage Leslie matrix projection…

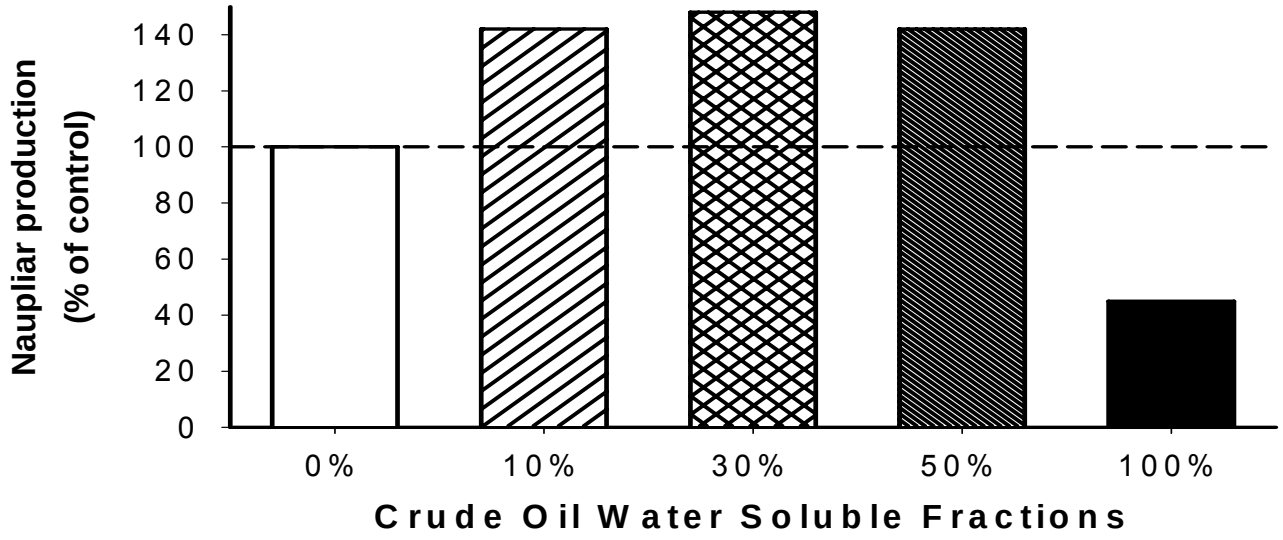
$N_e(t+1)$ (embryos)	=	P_{ee}	0	0	0	F_{en} [fecundity]	$N_e(t)$
$N_n(t+1)$ (nauplii)		P_{en} [hatch]	P_{nn}	0	0	0	$N_n(t)$
$N_c(t+1)$ (co-dites)		0	P_{nc} [growth]	P_{cc}	0	0	$N_c(t)$
$N_f(t+1)$ (virgins)		0	0	P_{cf} [sex ratio]	P_{ff}	0	$N_f(t)$
$N_g(t+1)$ (gravids)		0	0	0	P_{fg} [fertility]	P_{gg}	$N_g(t)$

X

Note: One Leslie matrix projection per control and toxicant concentration; then compared…

3-Generational Production by 10 Naups

NIST



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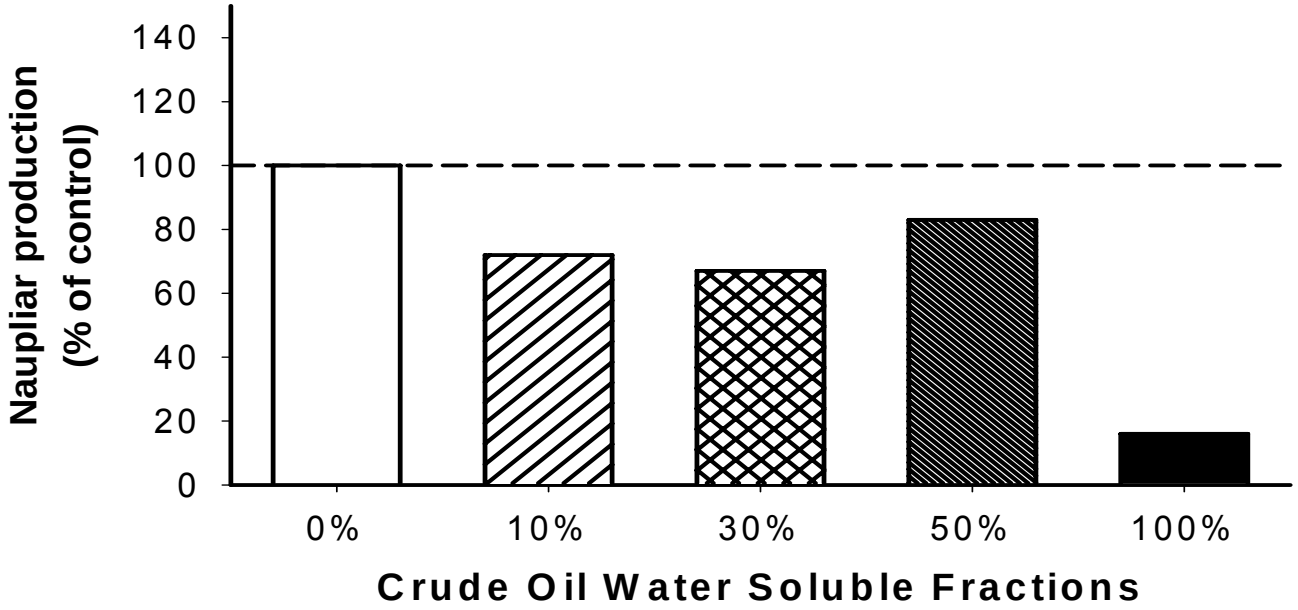

LESLIE MATRIX MODEL

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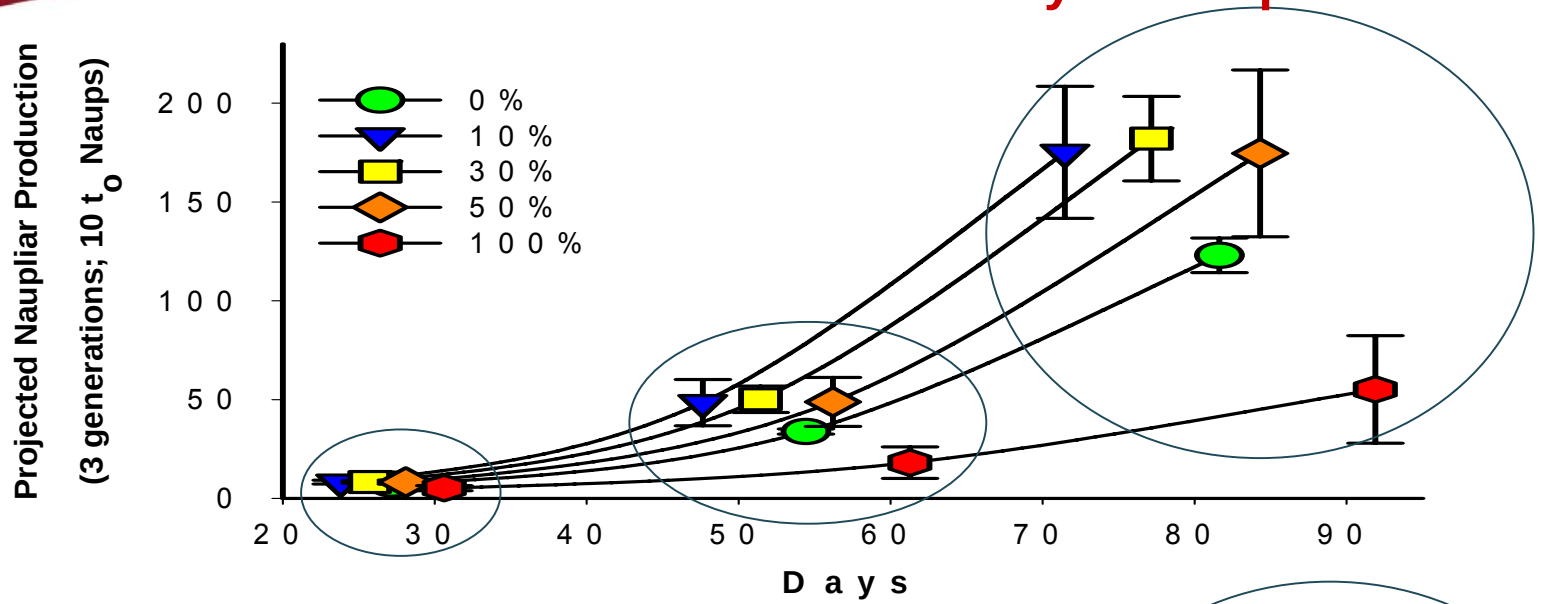
3-Generational Production by 10 Naups

LESLIE MATRIX MODEL



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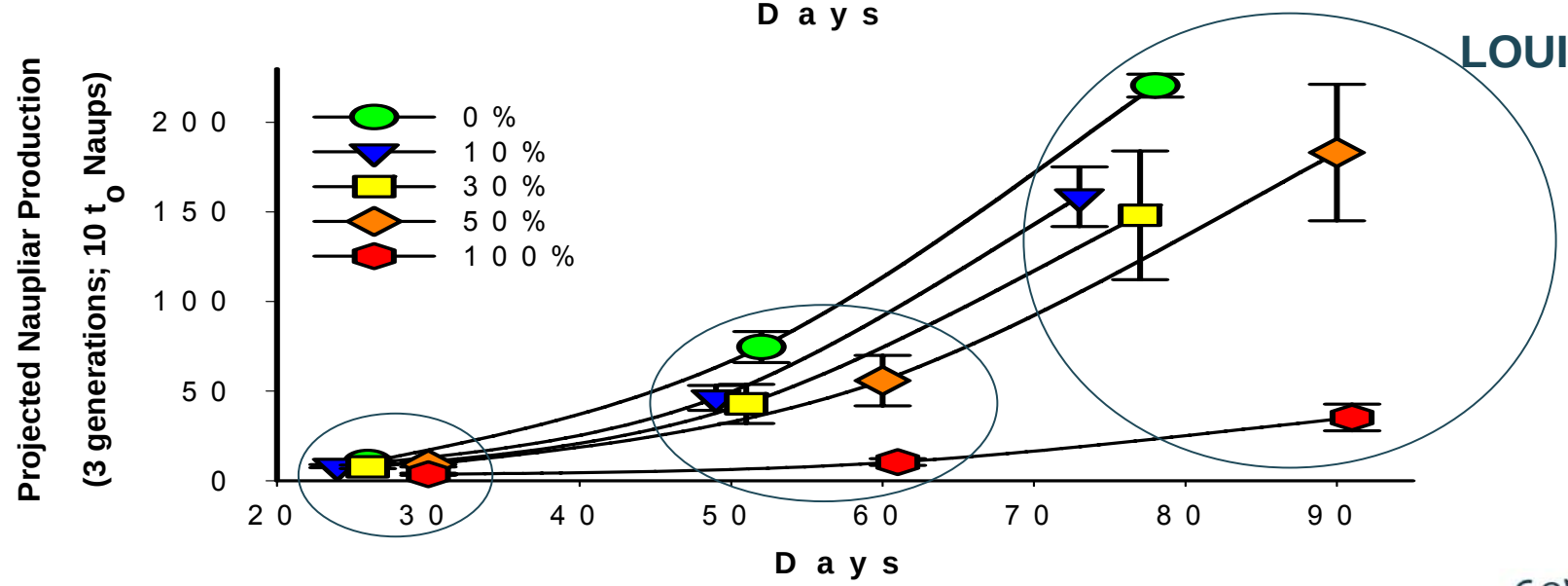

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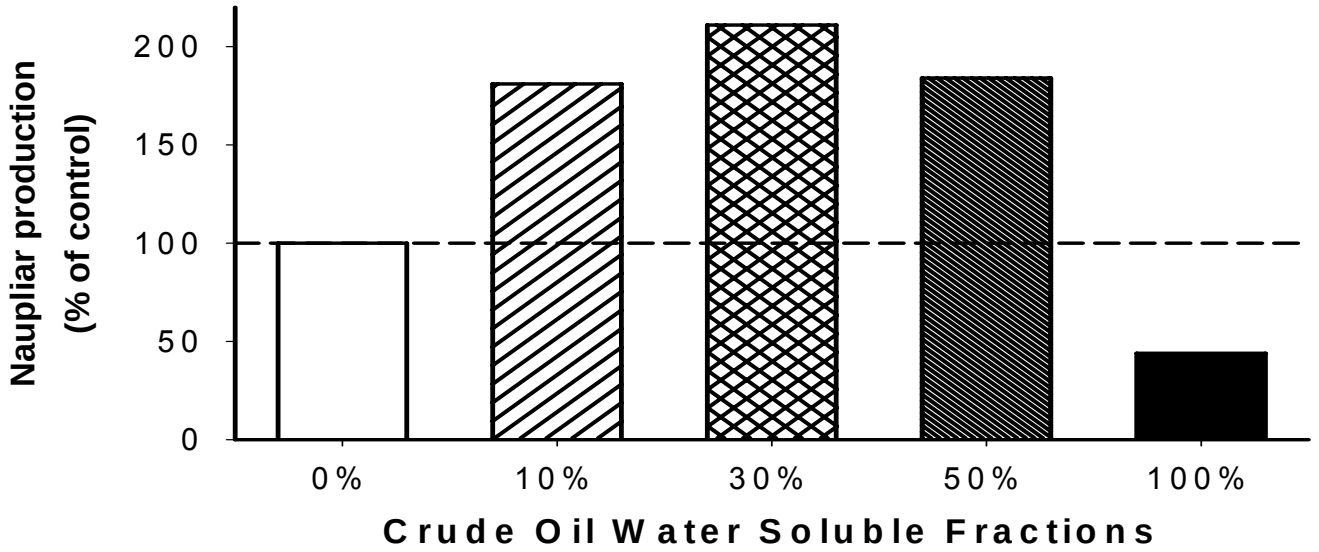
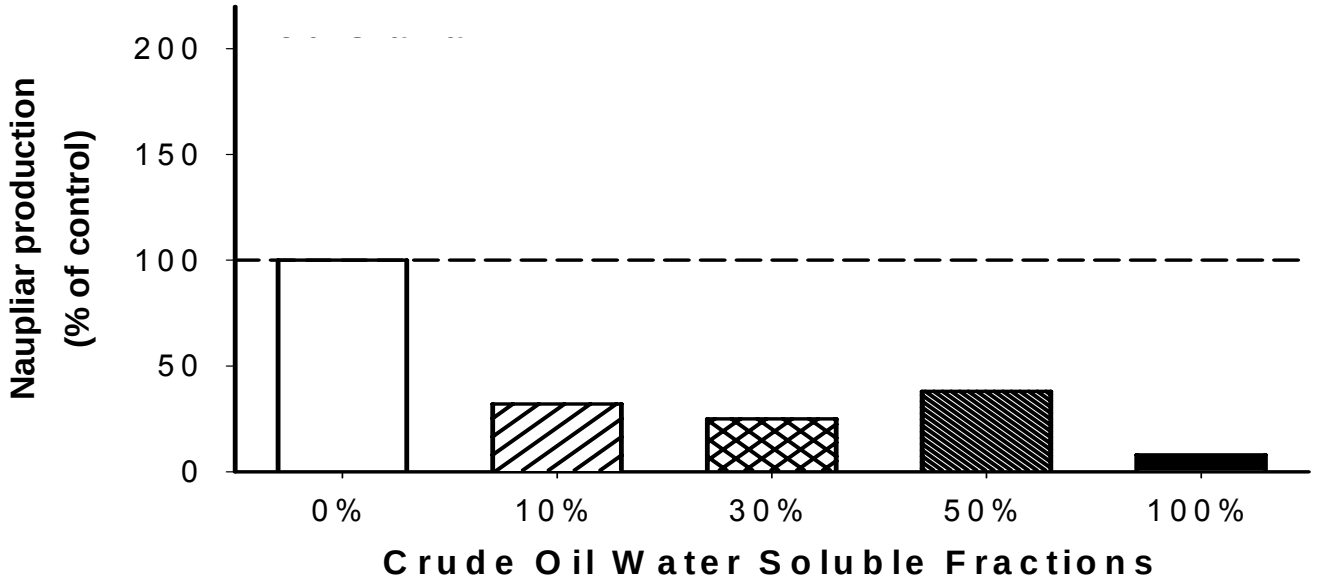
3-Generational Production by 10 Virgins

NIST



LOUISIANA



LESLIE MATRIX MODEL

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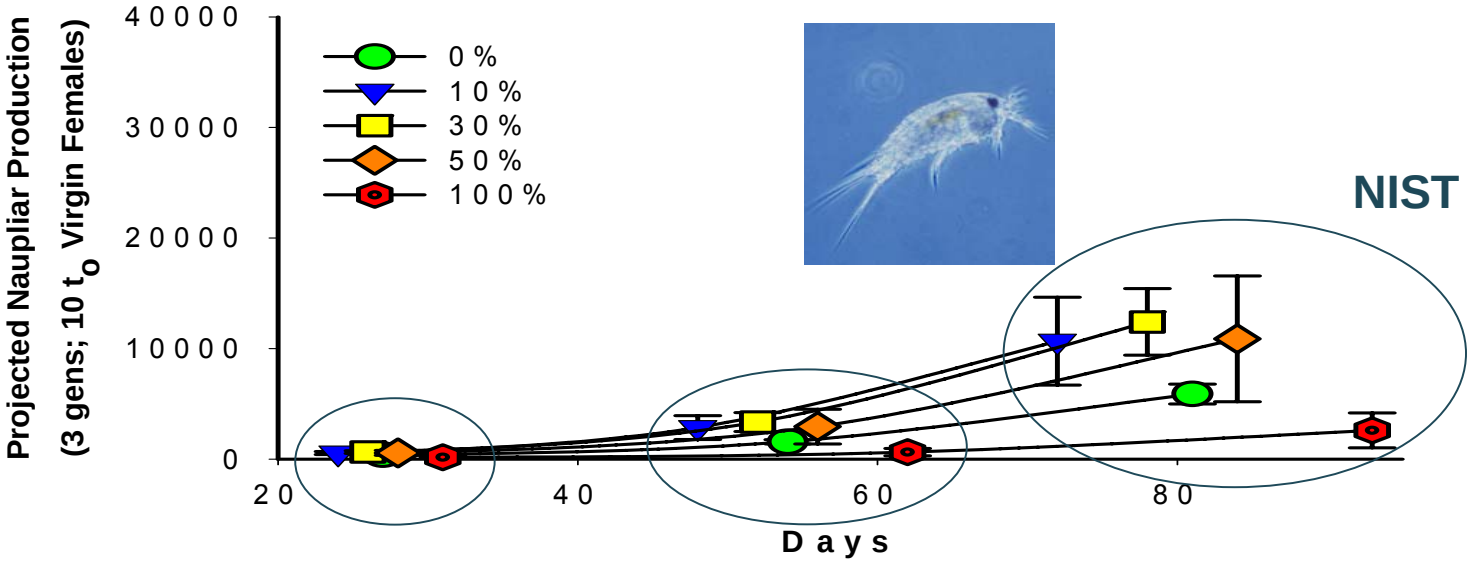

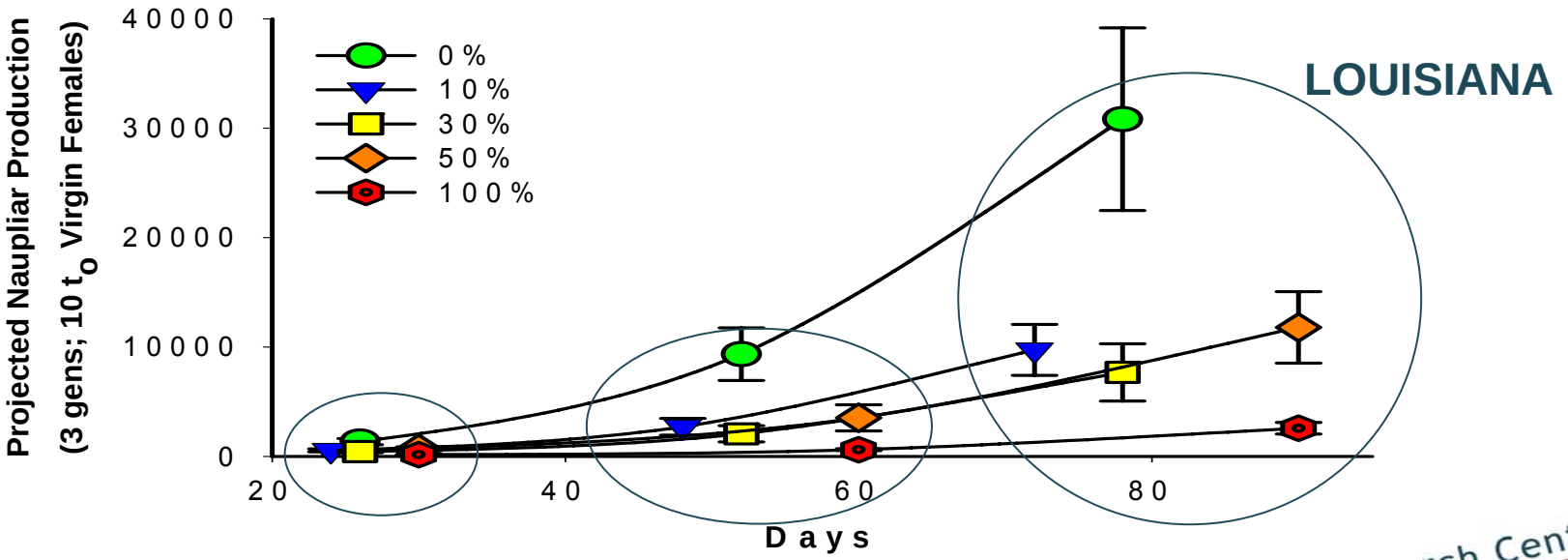

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Louisiana 50%-WAF Effects on Time to Carrying Capacity K

Scenario	Control	50%- WAF
Leslie-Matrix Time Steps to K	14.2	15.3
Time Steps per Generation	5	5
Mean generation time (days)	26.1± 2.3	30.0± 1.7
Estimated time to K (days)	74.1	91.9
Logistic Regression Coefficient (R^2)	0.995	0.998

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Predicted 3rd generation population sizes and finite rates of increase (λ) from our running *historic* control Leslie matrix when all vital parameters are simultaneously reduced by 0%, 15%, 30% and 45%.

Model Reduction (%)	Leslie-Predicted Population Sizes at F_3	Finite Rates of Population Increase (λ)
0% (control)	19936± 888	1.57
-15%	8849 ± 4928 (55.6% drop)	1.33
-30%	492 ± 424 (97.6% drop)	1.10
-45%	14 ± 22 (99.9% drop)	0.86

Conclusions regarding WAF copepod bioassay & NIST Referencing Approach

SL WAF (not surprisingly) showed much stronger individual and population-level effects than the less aromatic, relatively non-toxic NIST standard (Kuwaiti source). At higher dose levels, both crudes yielded strong reproductive toxicity that manifested at pairs' "ability to produce a clutch", and also in net production.

Leslie matrix projections of all lifetable parameters in an integrated model showed dramatic population reductions for the SL WAF relative to Controls and the NIST reference. UV-enhanced toxicity must be given consideration in test design and models.

We have observed high bioassay to bioassay temporal consistency for the NIST WAF reference. Since NIST maintains the highest consistency and integrity of reference standard materials in the world, the ability to use a NIST reference as a comparison to any crude oil of interest to NOAA (or EPA) is a real strength of this approach.

The OECD is validating this bioassay presently for Tier II screening of EDCs by Spring 07.

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Acknowledgement

Funding for this project was provided by the Coastal Response Research Center

www.crrc.unh.edu

Research Planning Inc. supplied Louisiana Crude

Summary of NIST WAF lifecycle effects:

Bioassay Endpoints	WAF- Effects Observed (p<0.05)
Naupliar survival	100% WAF (-)
Development from N-to-Copepodite	10% (+), 50% (-), 100% (-)
Copepodite survival	NONE
Development from C-to-Female	100% (-)
Development from C-to-Male	30% (-), 100% (-)
Sex ratios	NONE
Fertilization success	NONE
Brood size thru 2 clutches	NONE
Hatching success	100% (-)
3-gen population size	10% (+), 30% (+), 50% (+), 100% (-)
UV-mediated survival rate	30% (-), 50% (-)
UV mediated development rate	30% (-), 50% (-)

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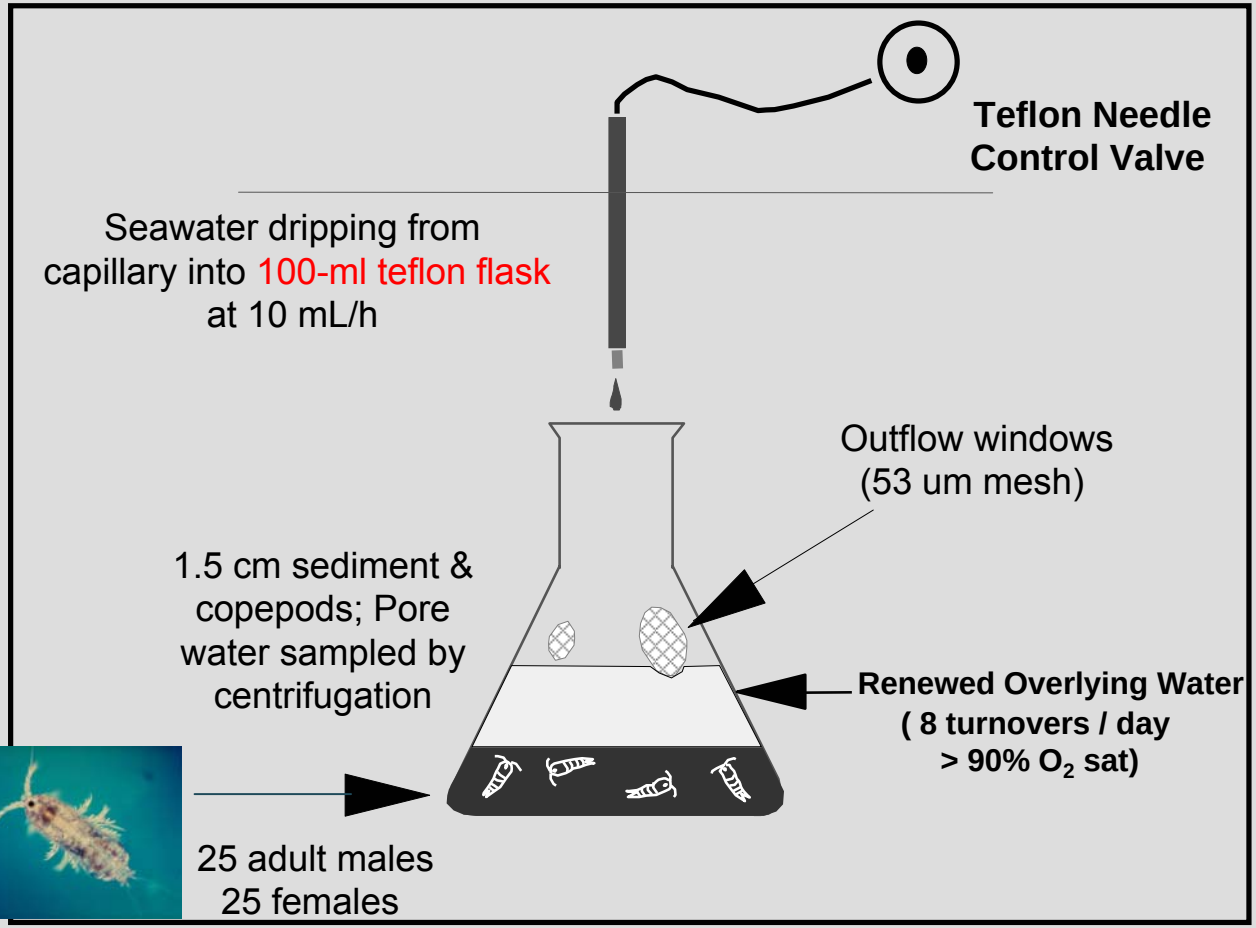
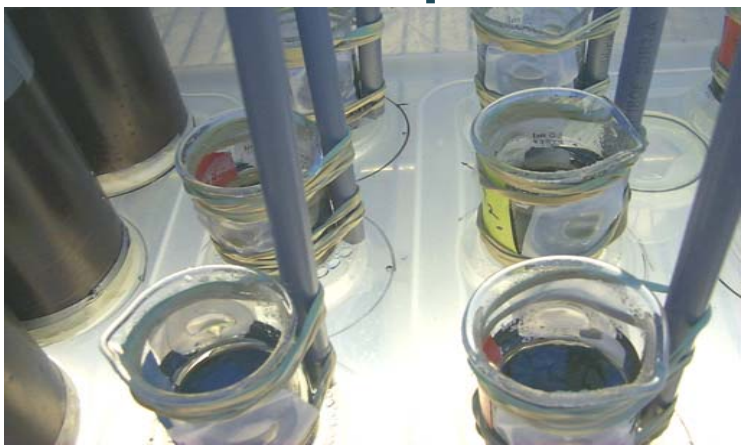
OK, this copepod is an infaunal sediment burrowing/ingesting beast…

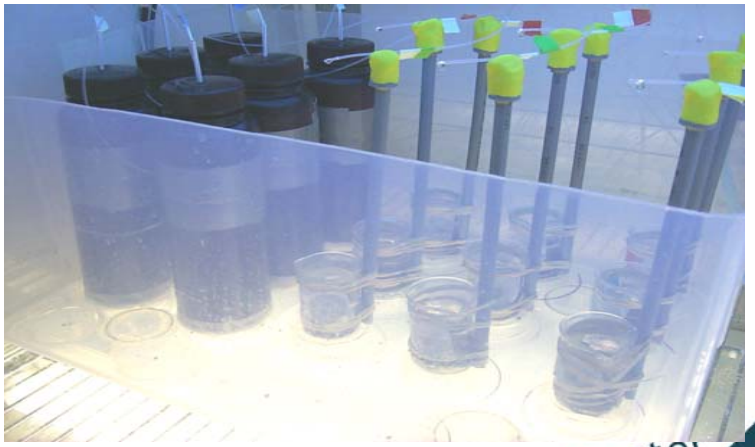
What about sediment-associated PAH effects?

Sediment Bioassay Chamber Design



Approach for Copepod UV / non-UV Sediment Exposures





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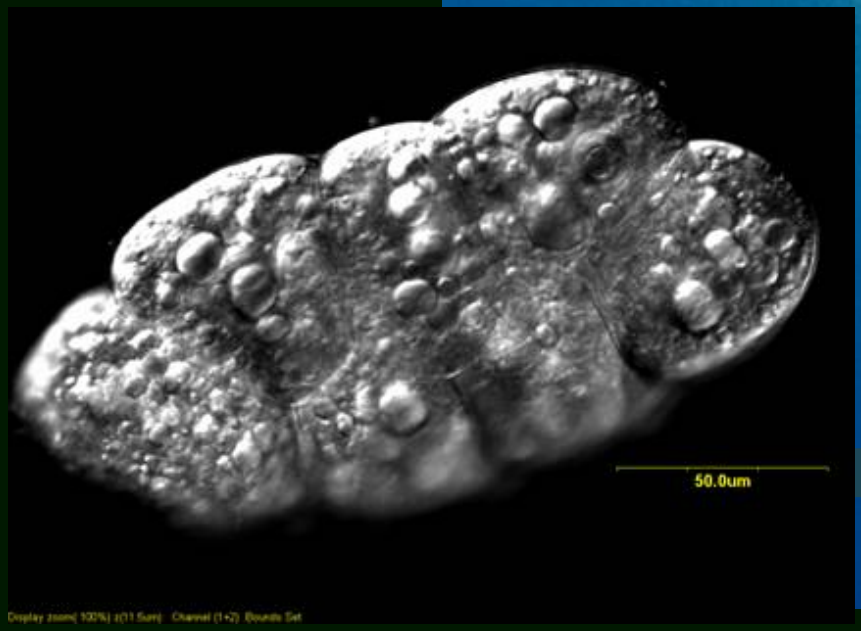
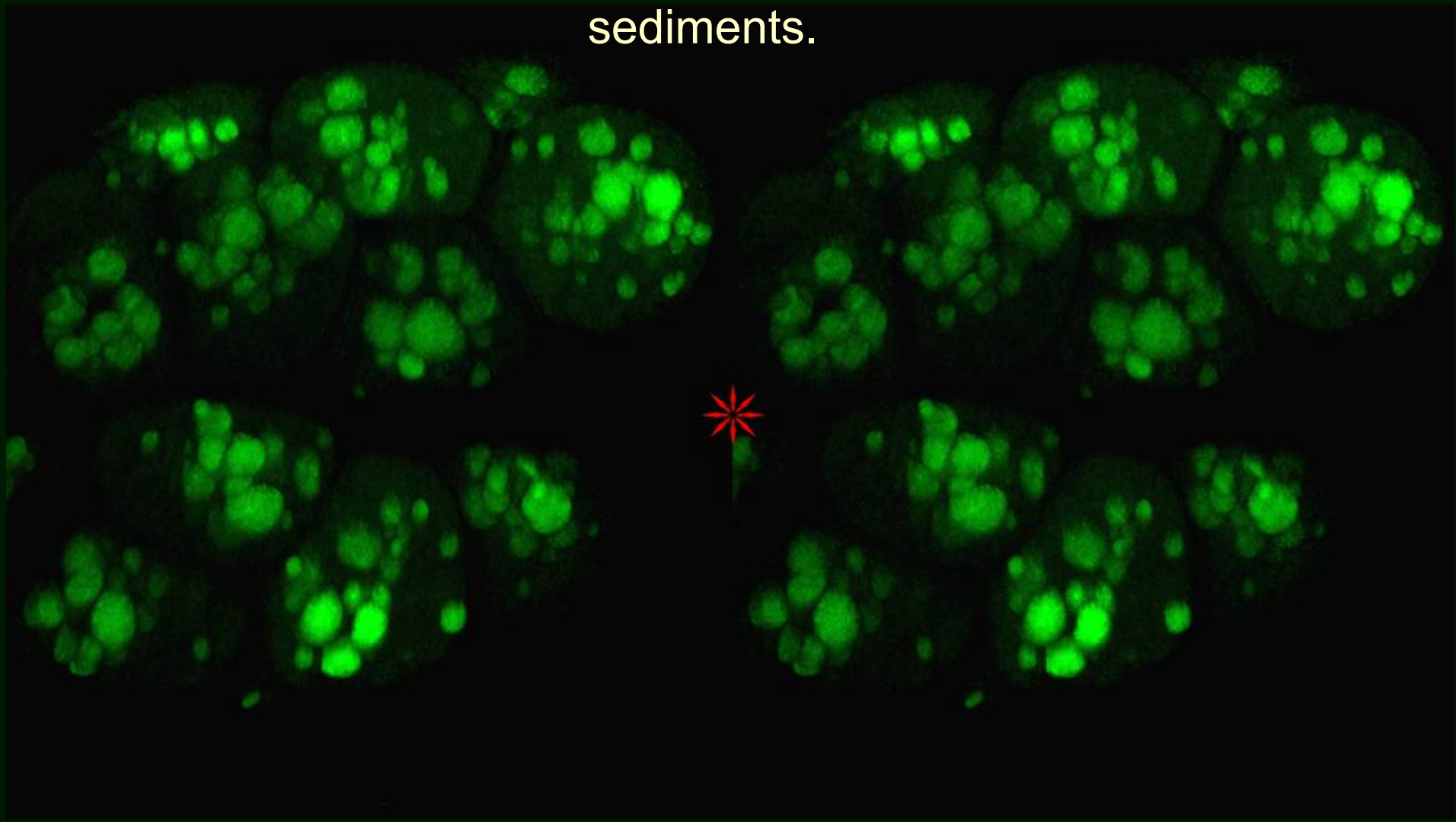
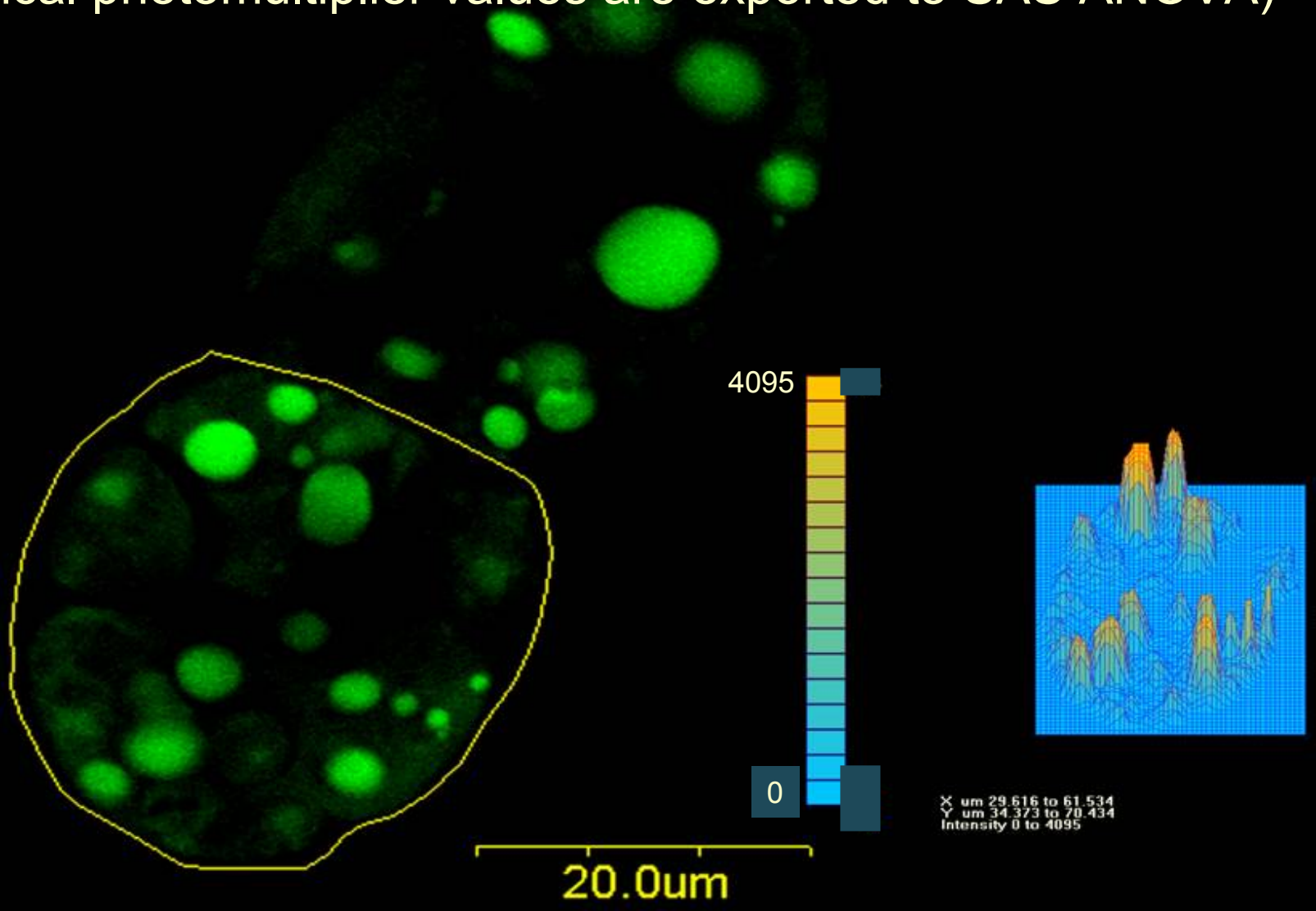
Amphiascus tenuiremis
gravid female

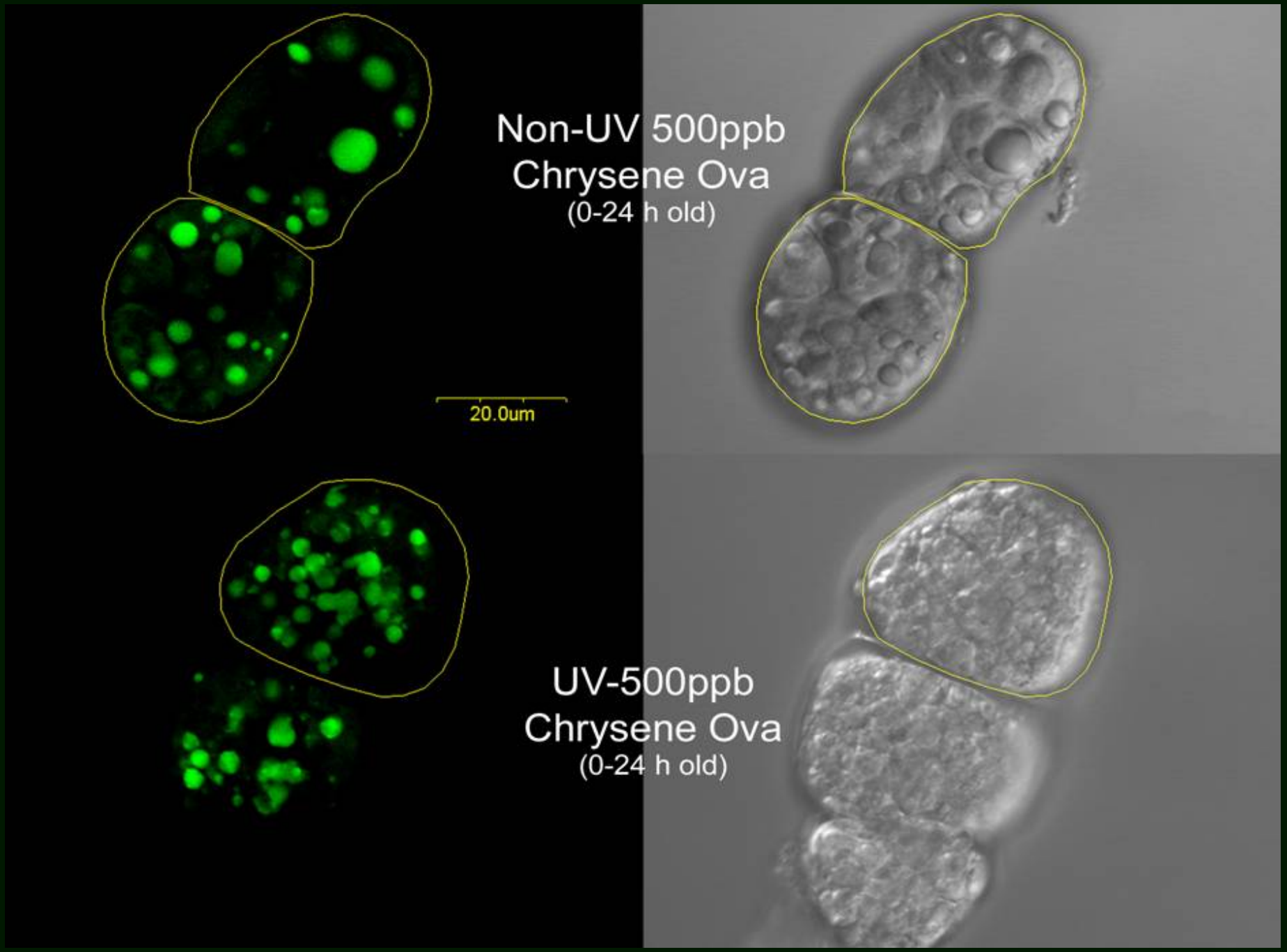


Stereopair images of BODIPY-stained embryos from a non-UV exposed *Amphiascus* female reared from nauplius to adult in 2500 ppb chrysene-spiked sediments.



FLUOVIEW plot of BODIPY fluorescence in a single *Amphiascus* egg.
(Numerical photomultiplier values are exported to SAS ANOVA)




Non-UV 500ppb
Chrysene Ova
(0-24 h old)
20.0um
UV-500ppb
Chrysene Ova
(0-24 h old)

Mean lipovitellin fluorescence for embryos produced in each chrysene treatment. Bars with dissimilar letters are significantly different (P>0.95). Note that no gravid females were produced in UV-2500 treatment.