Development of Receptor- to Population-Level Analytical Tools for Assessing Endocrine Disruptor Exposure in Wastewater-Impacted Estuarine Systems

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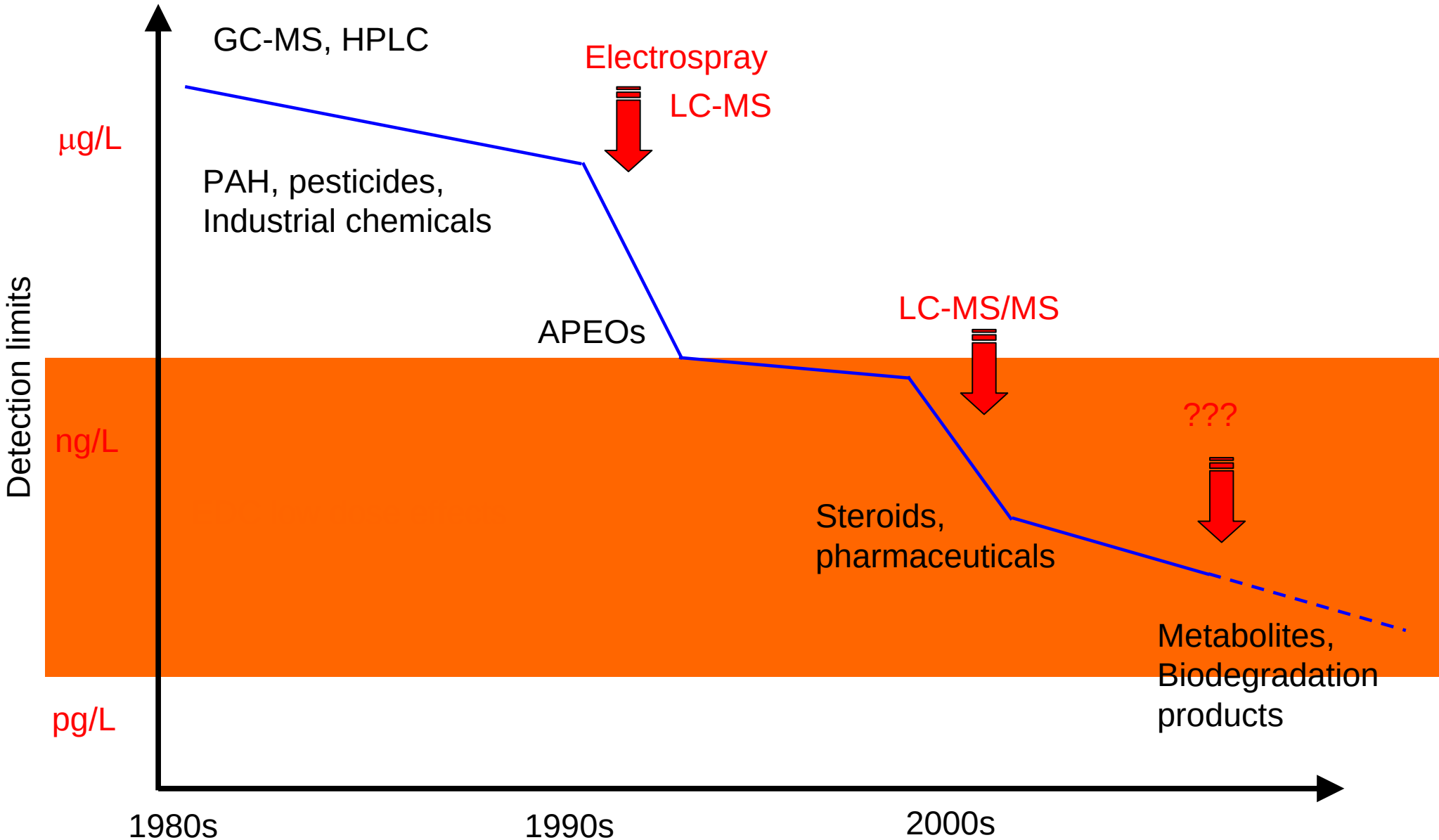
Grand challenges for assessing EDC exposure in the aquatic environment

- Assessing identity – this is not well constrained, we have a few well defined classes (steroids, some industrial chemicals), what about metabolites (e.g. APEOs)?
- Assessing sources – highly diverse and dynamic both spatially and temporally (WWTP, CAFOs, agricultural runoff)
- Assessing exposure levels – the analytical chemistry is challenging – many EDCs are present at low concentrations and are highly polar molecules
- MIXTURES! – the most difficult challenge, determining which components of complex chemical mixtures contribute to EDC activity

The analytical chemistry continuum



Analytical methods for EDCs

- HPLC-MS/MS – this has become the “gold standard” for many EDCs (high sensitivity, amenable to polar compounds)
- For maximum sensitivity, these analyses have typically been VERY focused (e.g. triple quadrupole MS/MS systems).
- NEEDED: broadband, sensitive, mode-of-action based methods for surveying exposure to EDCs in mixtures

Study objectives

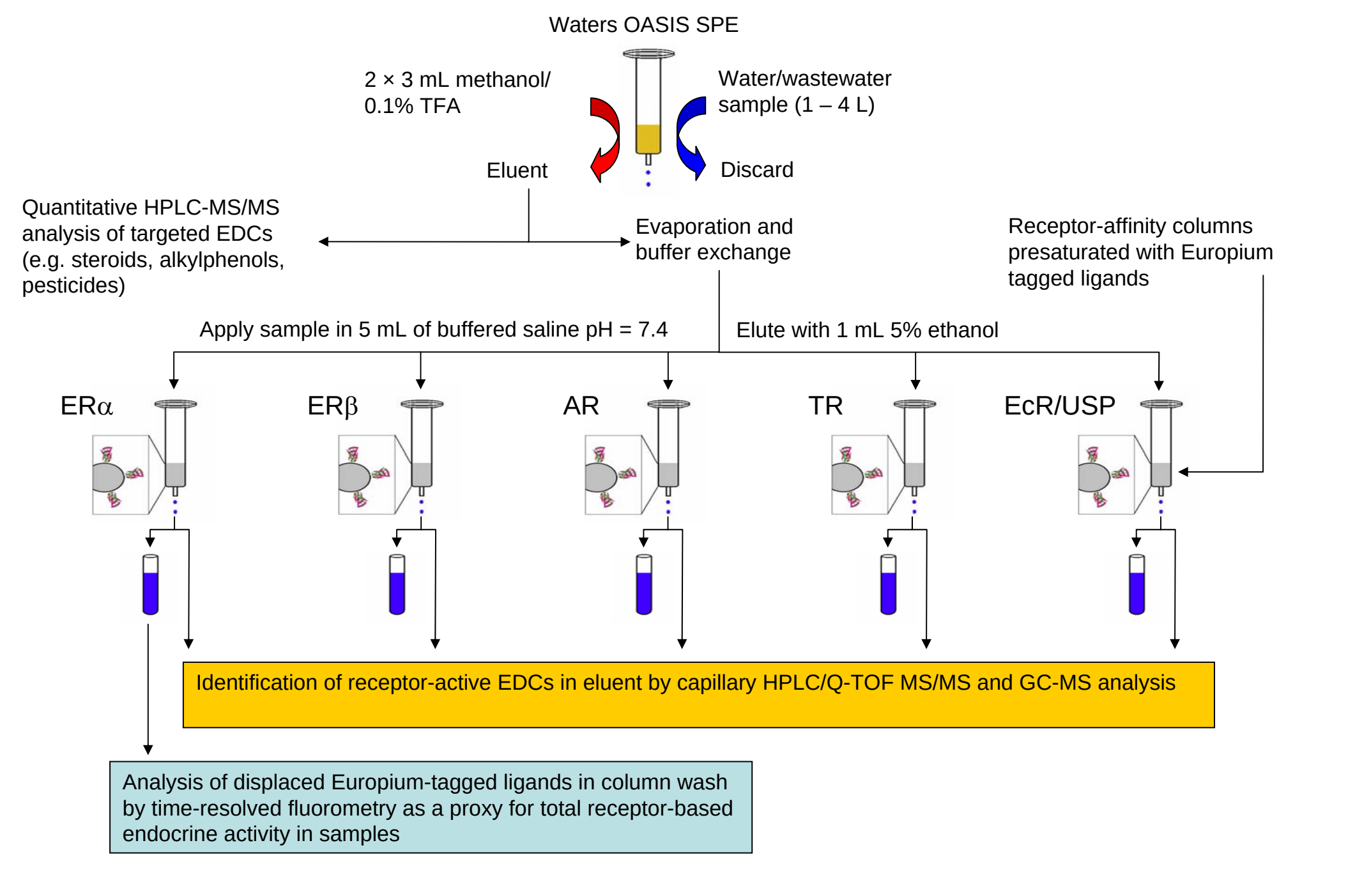
1. Develop nuclear hormone receptor-affinity extraction techniques as tools for isolating EDCs from complex wastewater mixtures
2. Apply those methods in combination with high-performance mass spectrometry for activity-directed analysis of EDCs in wastewater and estuarine receiving waters on the SC coast
3. Utilize sensitive vertebrate (zebrafish) and invertebrate (copepod) EDC-exposure laboratory assays to link exposure measurements to biological effects
4. Apply novel biomolecular endpoints to assess EDC exposure in field populations of sensitive meiobenthic invertebrates in wastewater-impacted estuarine environments.

Nuclear hormone receptors as bioanalytical tools

- Nuclear receptor activity is implicated in many EDC modes of action (e.g. estrogenicity, androgen agonists)
- These proteins form the basis of many common EDC screening tools (e.g. E-screen, YES/YAS, transient transfection assays, transgenics).
- They establish a clear, molecular link between EDCs and the biochemical pathways by which these compounds induce effects
- Because of the direct interaction between nuclear receptors and their ligands (or xeno-ligands), these biomolecules can be used as affinity reagents for analyte capture

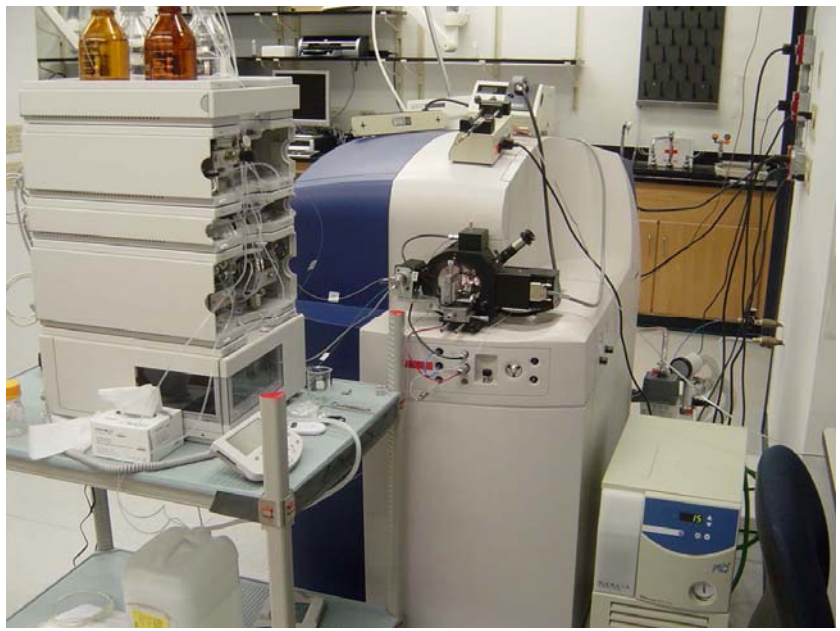
Receptor-affinity extraction

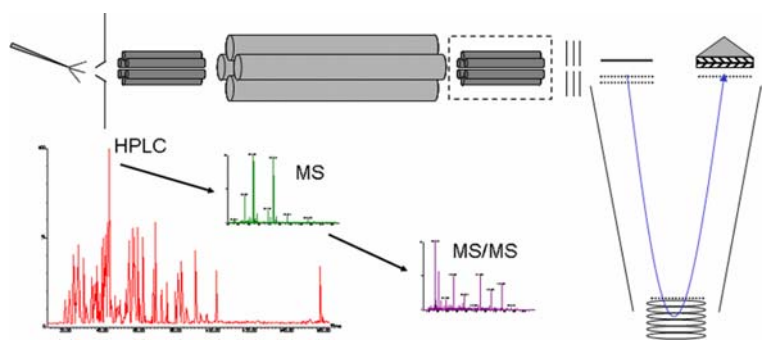
- Similar in concept to immunoaffinity chromatography – relies on high specificity/selectivity molecular interaction to isolate target analytes from a mixture prior to analysis
- Recombinant proteins (ligand binding domains of nuclear receptors)
  - Estrogen receptor (α/β isoforms, human)
  - Androgen receptor (human)
  - Thyroid hormone receptor (human)
  - Ecdysteroid/Ultraspiracle receptor sysem (mysid)
- Proteins are cloned, expressed in bacterial vectors, and purified chromatographically prior to immobilization on an inert solid phase support


Waters OASIS SPE
2 × 3 mL methanol/
0.1% TFA
Water/wastewater
sample (1 – 4 L)
Eluent
Discard
Quantitative HPLC-MS/MS
analysis of targeted EDCs
(e.g. steroids, alkylphenols,
pesticides)
Evaporation and
buffer exchange
Receptor-affinity columns
presaturated with Europium
tagged ligands
Apply sample in 5 mL of buffered saline pH = 7.4
Elute with 1 mL 5% ethanol
ERα
ERβ
AR
TR
EcR/USP
Identification of receptor-active EDCs in eluent by capillary HPLC/Q-TOF MS/MS and GC-MS analysis
Analysis of displaced Europium-tagged ligands in column wash
by time-resolved fluorometry as a proxy for total receptor-based
endocrine activity in samples

Analysis of EDCs by HPLC-QTOF-MS

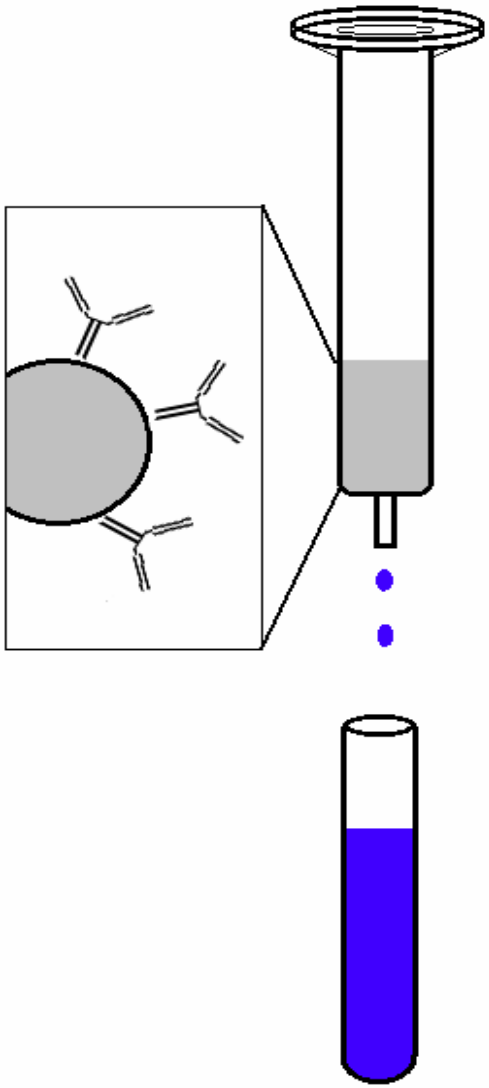
- High resolution separations by capillary HPLC for maximum sensitivity
- TOF mass spectrometry provides high mass measurement accuracy for elemental formula elucidation
- High resolution MS/MS capability allows structural analysis of non-target analytes
- Quantitative analysis can be performed in parallel by triple-quadrupole mass spectrometry





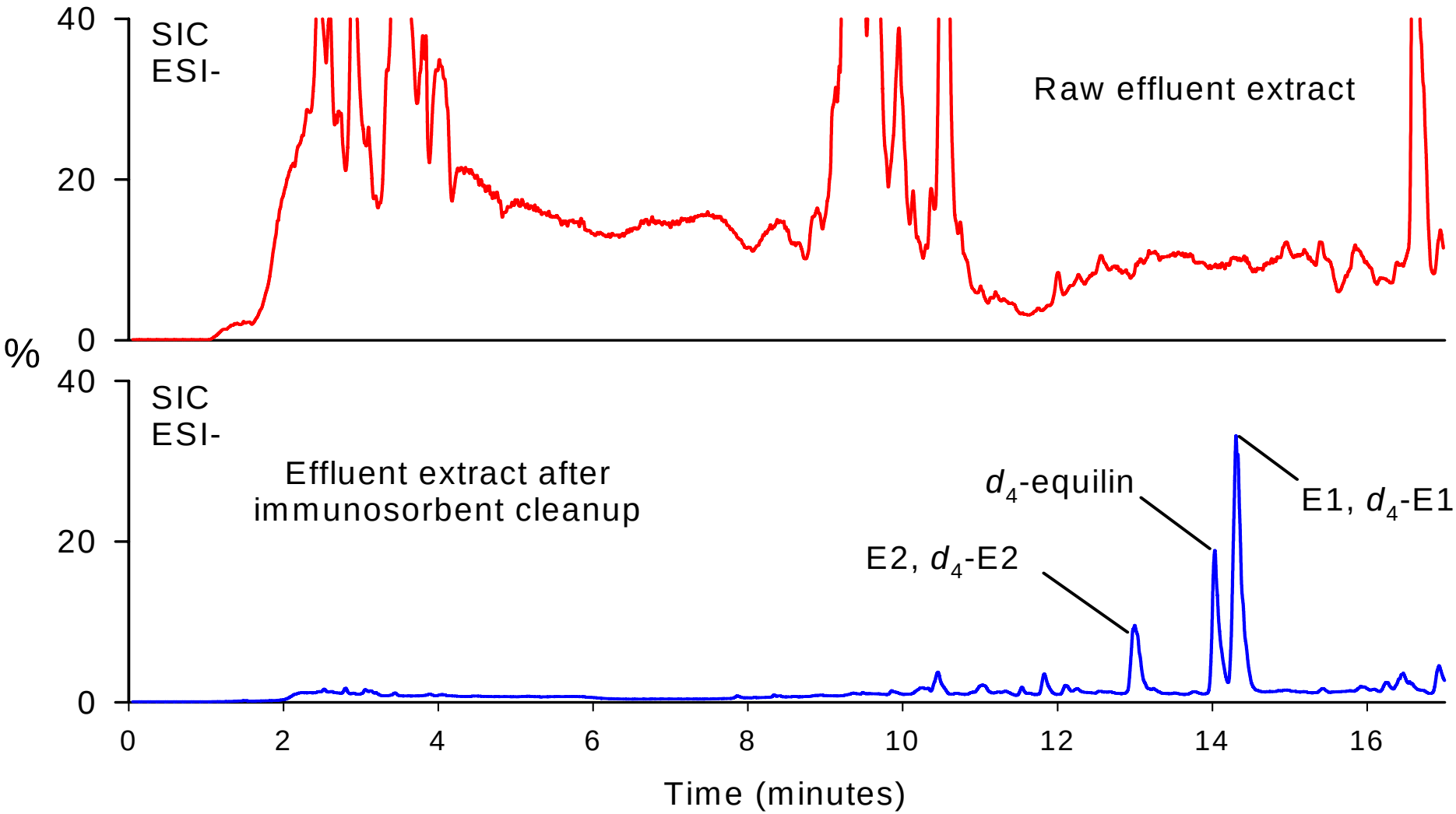
Proof-of-concept: immunoaffinity extraction and quantitative analysis of steroid estrogens in treated wastewater by HPLC-MS

Immunosorbent cleanup for steroid estrogens in wastewater



- Monoclonal antibodies are coupled to a solid support
- Wastewater extract (isolated by solid phase extraction) is passed through the cartridge
- Interferences are washed off the sorbent, while estrogens are selectively retained
- Estrogens are eluted in a single fraction
- The extract is ready for LC-MS analysis

HPLC-ESI-MS of estrogens in sewage effluent



Estrogen immunoaffinity-HPLC-ESI-MS method validation

	% Recovery (SD)*		Effluent conc. (ng/L) (SD)**		
Compound	effluent	spiked Milli-Q water	Plant #1	Plant #2	MDL (ng/L) in effluent
E2	107.0 (9.3)	100.4	0.77	6.44 (0.32)	0.18
E1	91.8 (2.1)	87.8	1.61	17.6 (0.2)	0.07
EE2	1.7 (1.3)	58.3	n.d.	n.d.	n.d.

* Calculated using d_4-labeled surrogate standards
**Based on triplicate measurements

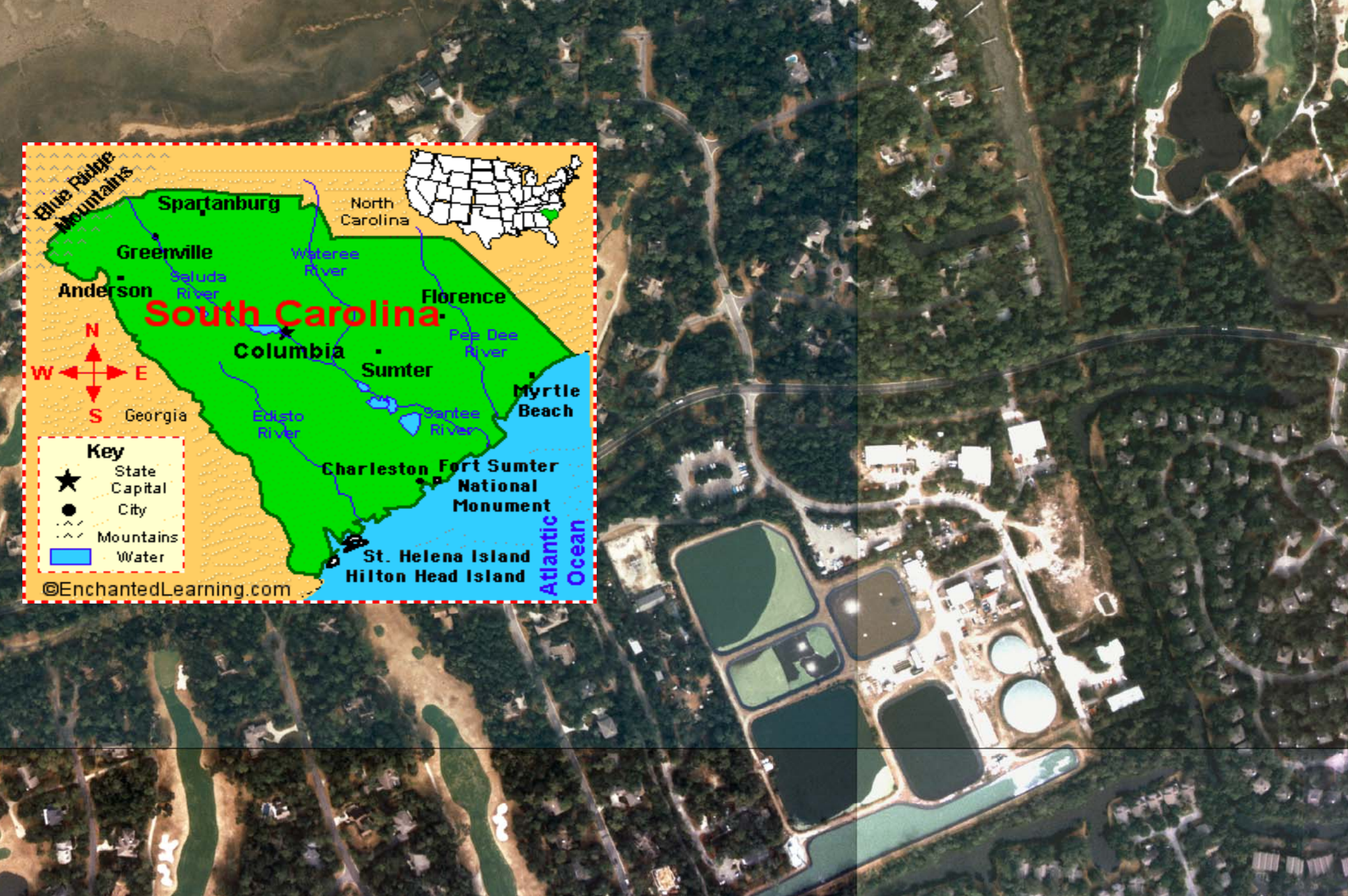
Work in progress

- Completed: cloning, expression, and purification of the ERα ligand binding domain – currently validating binding affinity using radioligand assay.
- In progress: cloning and expression of the human AR LBD in a thioredoxin fusion construct
- Next: EcR/USP, TR, and ERβ isoforms, initial immobilization studies and validation with simple EDC mixtures and with complex environmental mixtures from wastewater-impacted coastal environments in South Carolina

Coastal South Carolina sites for studying the impacts of wastewater-derived EDCs on estuarine systems

We have chosen two sites representing wastewater discharge regimes typical of SC coast

- Plum Island WWTP: conventional activated sludge plant discharging 36 MGD effluent into Charleston Harbor – sampling will include effluent and adjacent receiving waters
- Kiawah Island WWTP: discharges up to 10 MGD of treated effluent through land application (irrigation of golf courses) adjacent to tidal creeks – sampling will include effluent and runoff from the fairway turf
- Objective will be to characterize the endocrine-active components of these diverse wastewaters and receiving waters using the receptor-affinity columns described above in combination with more traditional targeted analysis for e.g. APEOs, steroids, pesticides


Blue Ridge Mountains
Spartanburg
North Carolina
Greenville
Wateree River
Saluda River
Anderson
Florence
South Carolina
Pee Dee River
Columbia
Sumter
N
W
E
S
Georgia
Myrtle Beach
Edisto River
Santee River
Key
State Capital
City
Mountains
Water
Charleston
Fort Sumter National Monument
St. Helena Island
Hilton Head Island
Atlantic Ocean
©EnchantedLearning.com

Bioassays for EDC activity will be utilized to link exposure measurements to biological effects

- Mock wastewaters will be reconstituted using qualitative and quantitative data generated from the receptor-affinity extraction/mass spectrometry analysis – goal is to reproduce endocrine activity of complex mixtures in controlled laboratory setting
- For estrogenic compounds, we will utilize an adult male zebrafish exposure, assaying vitellogenesis as the endpoint
- For ecdysteroid receptor-active compounds, we will test activity by using a novel estuarine copepod microplate assay that will allow testing of EDC effects from the molecular- to the population-level

Zebrafish bioassay

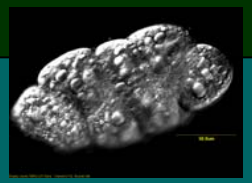
- Adult male zebrafish exposed to EDC mixture under constant flow for 48 – 72 hours
- Plasma collected at the end of the exposure
- Assay for vitellogenin using commercial ELISA sandwich assay (Biosense, Inc.)
- Sensitive, in vivo assay for estrogenic potency – well suited for linkage with ER receptor affinity columns

Copepod microplate bioassay & life-table methods for EDC molecular to population level assessment… survival, development rates, ecdysone/vitellin, reproductive success

Why use micro-crustaceans like copepods for ED risk assessment?

- To gauge chronic risks of EDCs to wildlife, we need population-relevant endpoints linked to ED processes: E.g., sex-specific fertility, mating success, egg production/quality, hatching success, $F_{0\text{-}N}$ pop growth rates, extinction risks, etc.
- Harpacticoid copepods give this kind of information quickly, which in turn can assist protection of other longer-lived invertebrates such as crabs, lobsters, and shrimps
- Laboratory assay results can be translated directly to field relevance – tools are appropriate for measurement of EDC effects in field populations

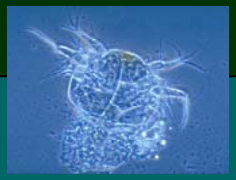
Discrete Lifestages of the meiobenthic copepod *Amphiascus tenuiremis* at 25C in 96-well microplate culture (15-35S)



Embryo

2 days

hatch



Nauplius
(6 stages)

8 days

Copepodite
(5 stages)

7 days

Adult
Male
or Female

1 day

mate

Adult
Gravid

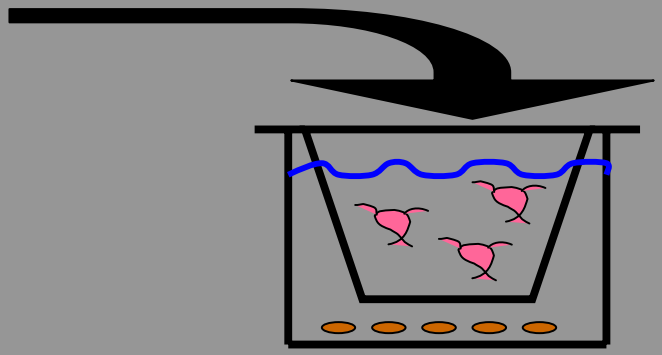
fecundity

- Lifecycle = 17-18 days
  Egg to Egg
- Avg. Life Expectancy = 47 ± 2 days
- Avg. Clutch = 6.2 ± 2 eggs
- 8-9 Clutches/Life

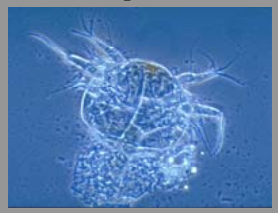
Note: Sediment lifecycle is ~20% faster.

How the copepod bioassay works..

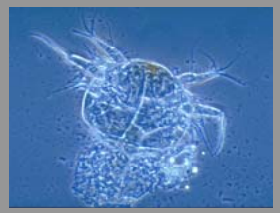
~ 250 gravid *A. tenuiremis*
(from lab stock mud cultures)



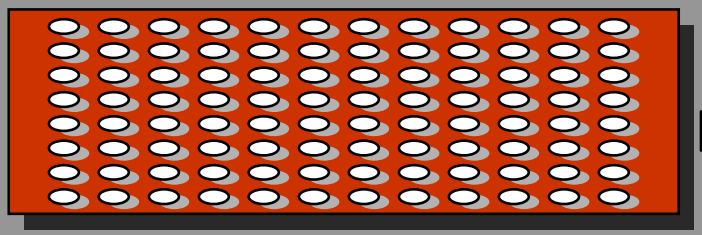
12-well plate with 75 μm meshed-well inserts;
Yields ~ 500 nauplii in < 24 hours



Amphiascus nauplius

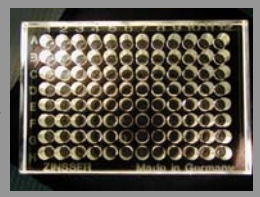


(n= 60-120/treatment over 3 plates/trtmnt)



96-well Costar® glass-lined or ultra-low attachment polystyrene microplate; 250 μl EDC suspect solution per 10 wells every other row.

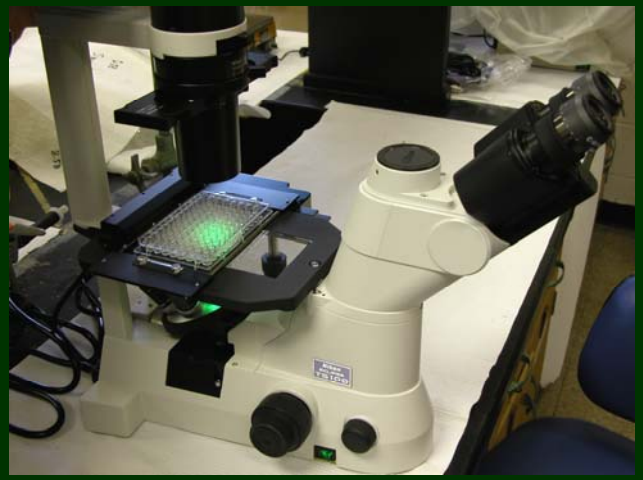
X-MATINGS



Life-cycle Endpoints:

- Survival & Molting Success
- Time to first Copepodite
- Time to Adult
- Sex Ratio of F_0
- Fertilization Success
- Clutch Size & Egg Quality
- Hatching Success
- Ecdysone, Vitellin, other biomarks.

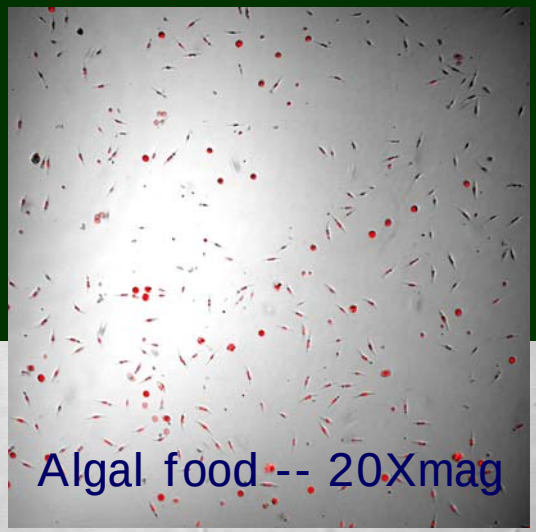
What the Microplate lifecycle Test looks like in practice…

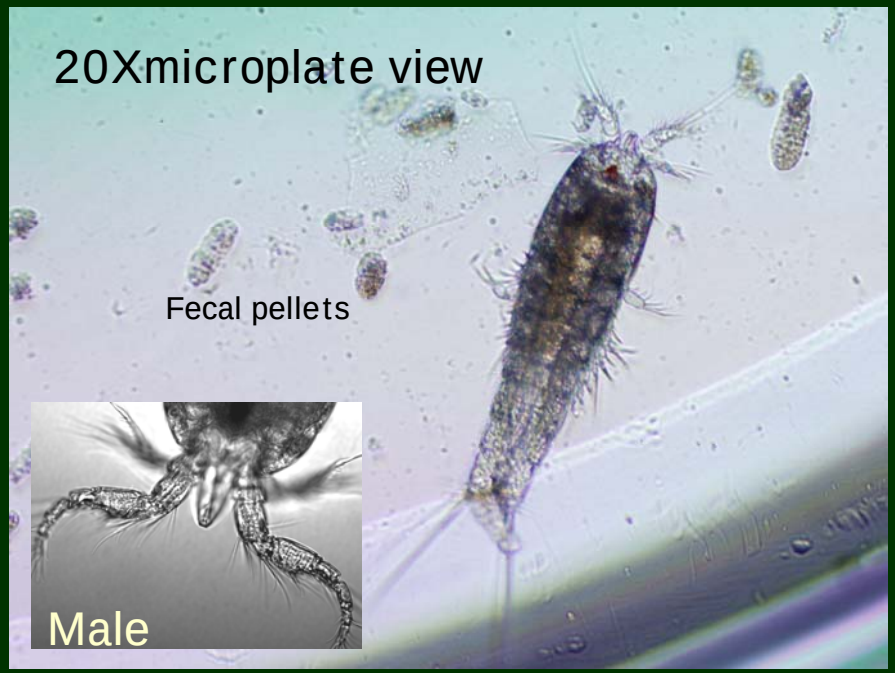


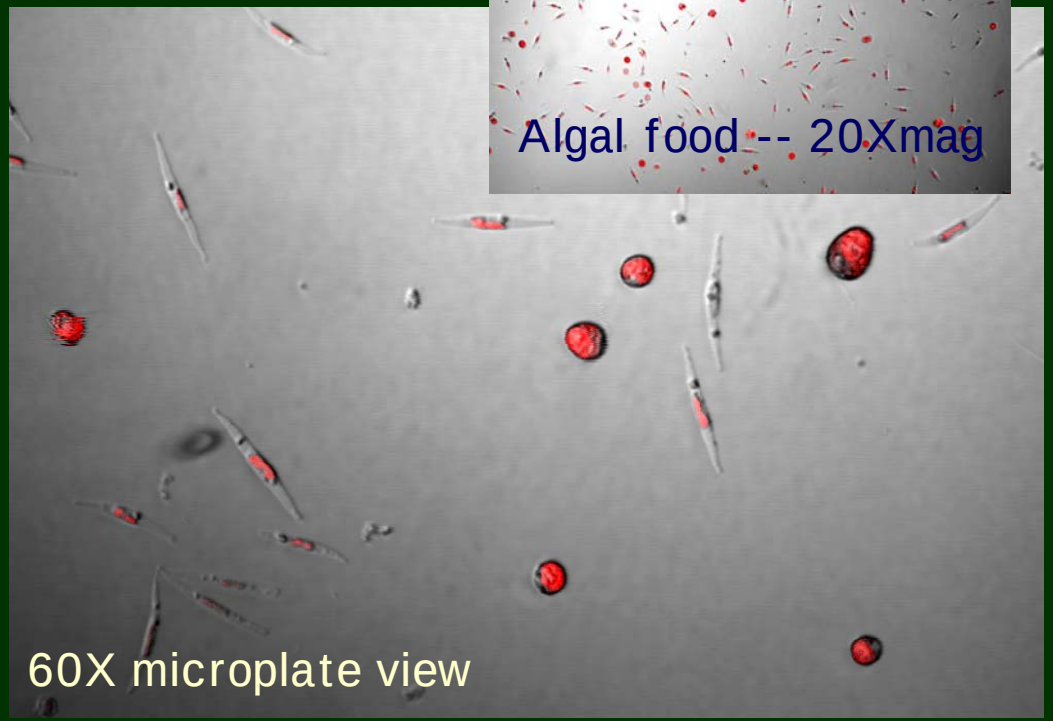
Designation: E 2317 – 04

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.







In 2003, the OECD 'Environment, Health and Safety Division' formed an *ad hoc* 'Expert Group on Invertebrate Testing' to validate "Tier II" ED-relevant lifecycle bioassays for their suitability to routine screening of suspected EDC's:

- Mysid shrimp 2-generation lifecycle test (USA, validated)
- Daphnia 2-generation sex-ratio test (Japan, in validation)
- *Acartia* (calanoid) copepod lifecycle test (Denmark, in validation)
- **Meiobenthic copepod microplate-based lifecycle test (USA/Sweden, presently completing validation)**
- Parthenogenic hydrobiid snail (*Potamopyrgus*) LC test (proposed, Germany)
- *Chironomus riparius* lifecycle test… (proposed, Germany)

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 (F1,F2)
- Hormonal/endocrine change (male lipovitellin, ecdysone @ virgin-adult, female vitellogenesis)
- Population growth parameters: λ, r, stable-age structure, extinction risk
- Multi-generational exposures with population modeling to n^{th} generations

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)

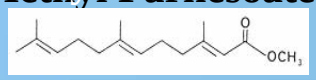
ENDPOINTS IN 'RED' ARE PROPOSED FOR NEW OECD Copepod Test Guideline


Lipovitellin in male

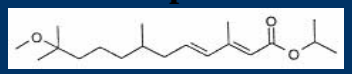
Effects of EDCs on Vitellin Expression

Juvenile Hormone Analogs

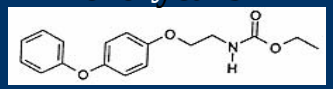
Methyl Farnesoate



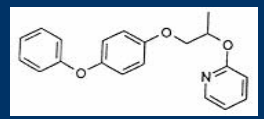
Methoprene



Fenoxycarb



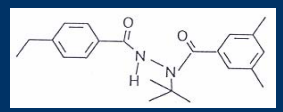
Pyriproxyfen



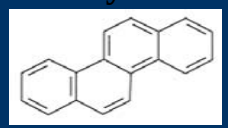
Female Y-organ

Ecdysteroid Analogs

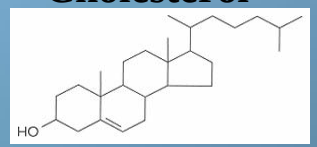
Tebufenozide



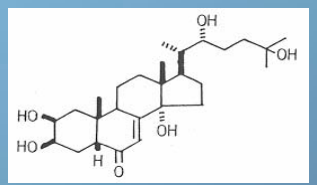
Chrysene



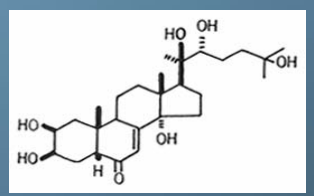
Cholesterol



Ecdysone



20-hydroxyecdysone



Yolk Granule

Vitellin

Lipid packaging and transport

Vtn mRNA

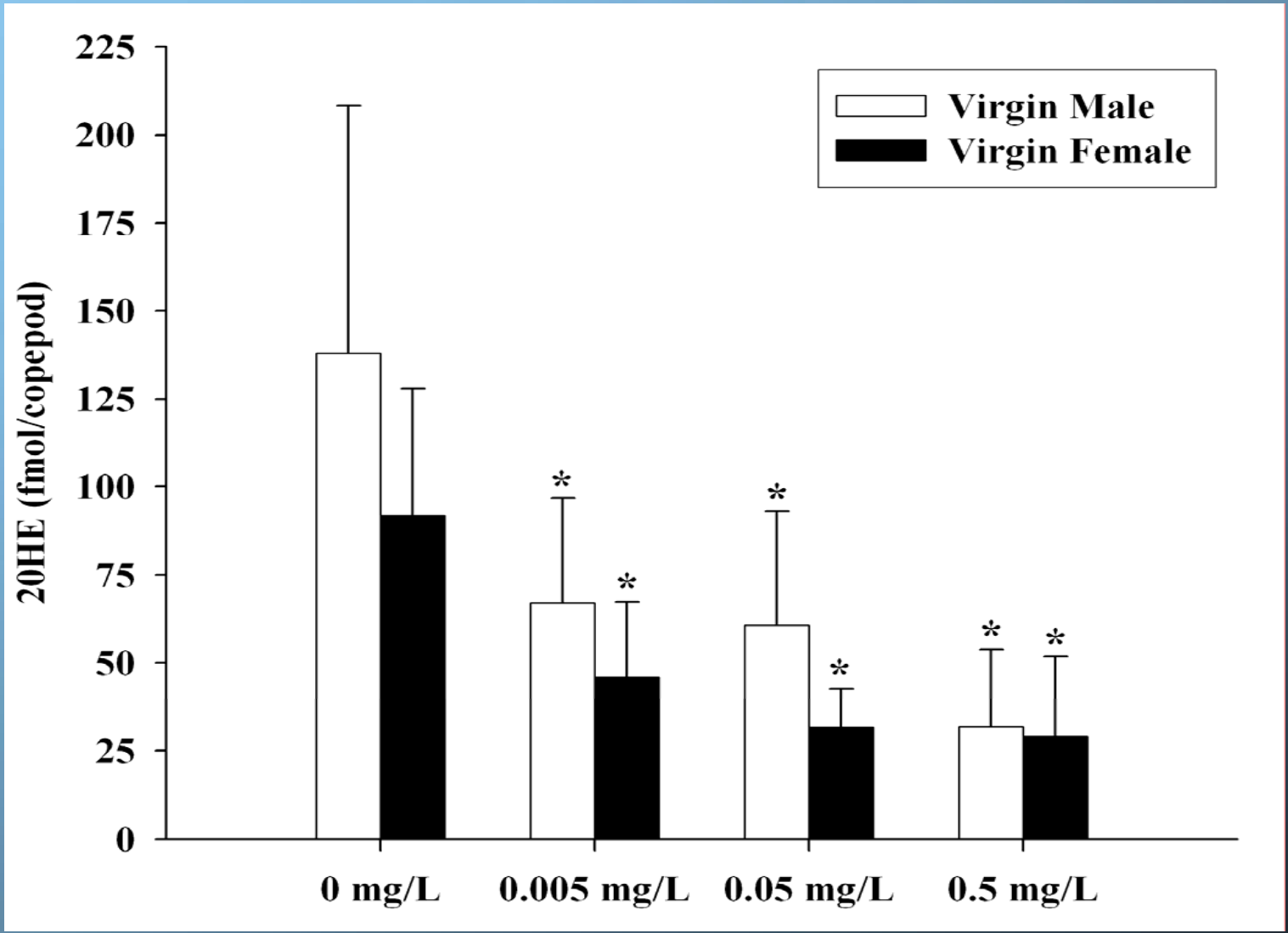
RNA Polymerase

EcR

Vitellin (Vtn) Gene

Ecdysone:Ultraspiracle Receptor Complex (EcR)

Fenarimol reared virgin-adult copepods showed significantly depressed 20HE

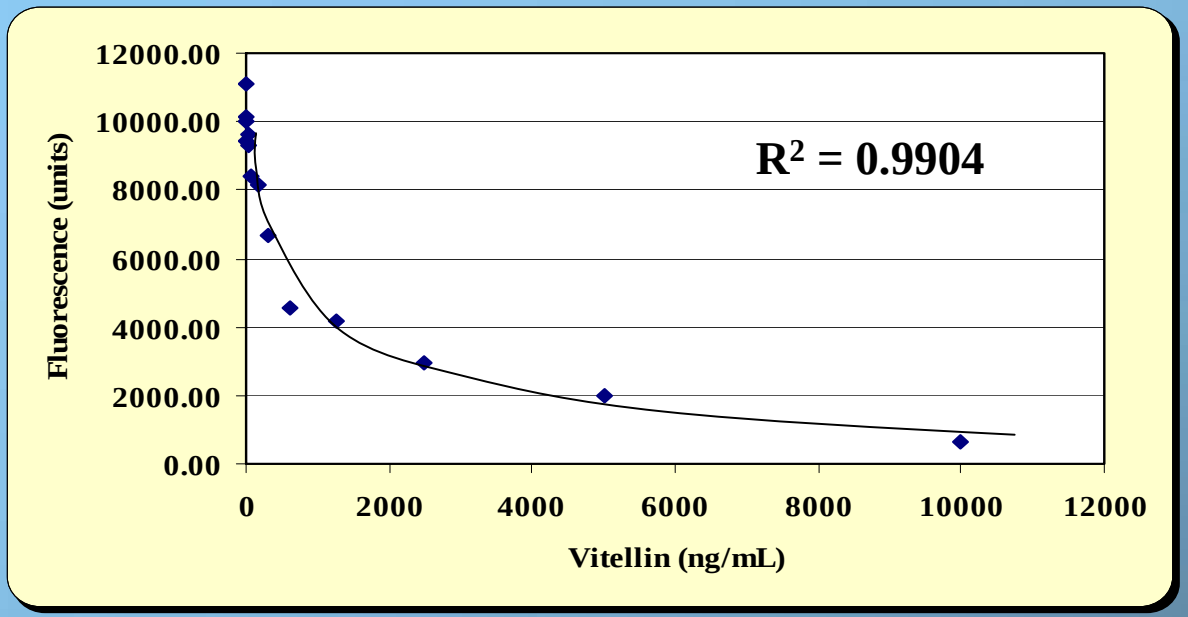


Significance of the Embryonic Crustacean Yolk Granules

Fuel for proper embryonic development and post-hatching survival.

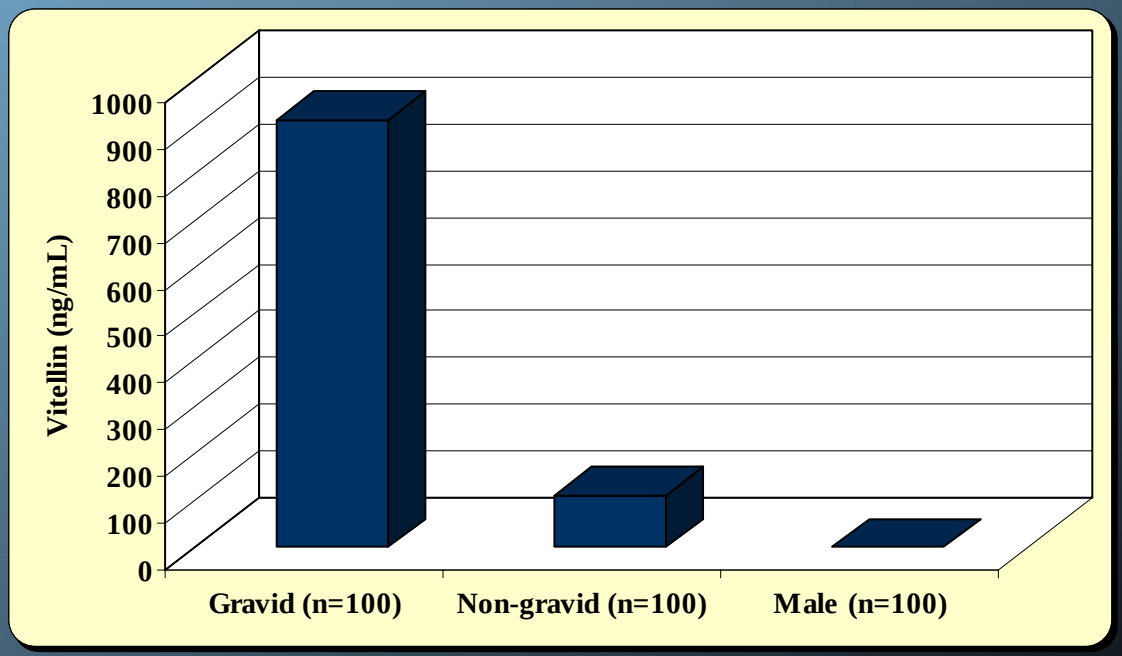
Maternal steroids (20HE) may be tightly bound to yolk granules for control of embryogenesis and early embryonic development; Secondarily, lipophilic toxicants can accumulate in yolk and possibly confer toxicity to embryos.

Copepod Indirect Competitive Anti-Vitellin ELISA



Standard Curve Dynamic Range:
2.4 ng/mL → 10,000 ng/mL

Vitellin Concentrations in *A. tenuiremis*

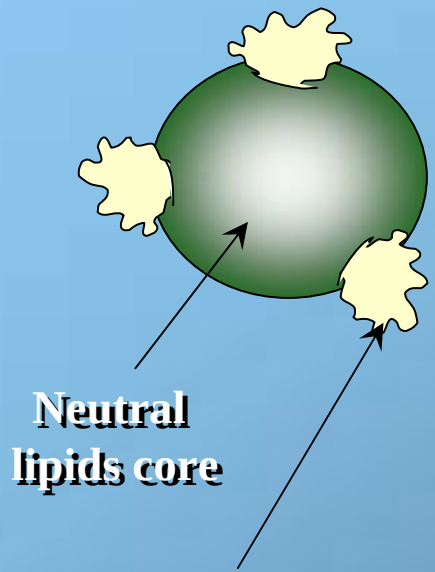


Immunolocalization of vitellin

Vitellin proteins are closely associated with the neutral lipid-filled yolk granules.

The neutral lipids probe, BODIPY 505/515, can be used for the qualitative and semi-quantitative assessment of vitellin as a biomarker of ED.

In Vivo Fluorescent Staining of Yolk Granules

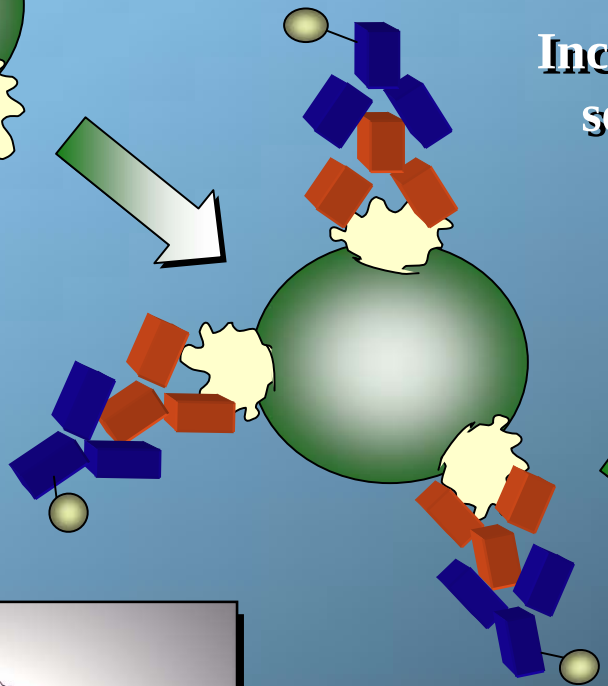


Neutral lipids core

Vitellin

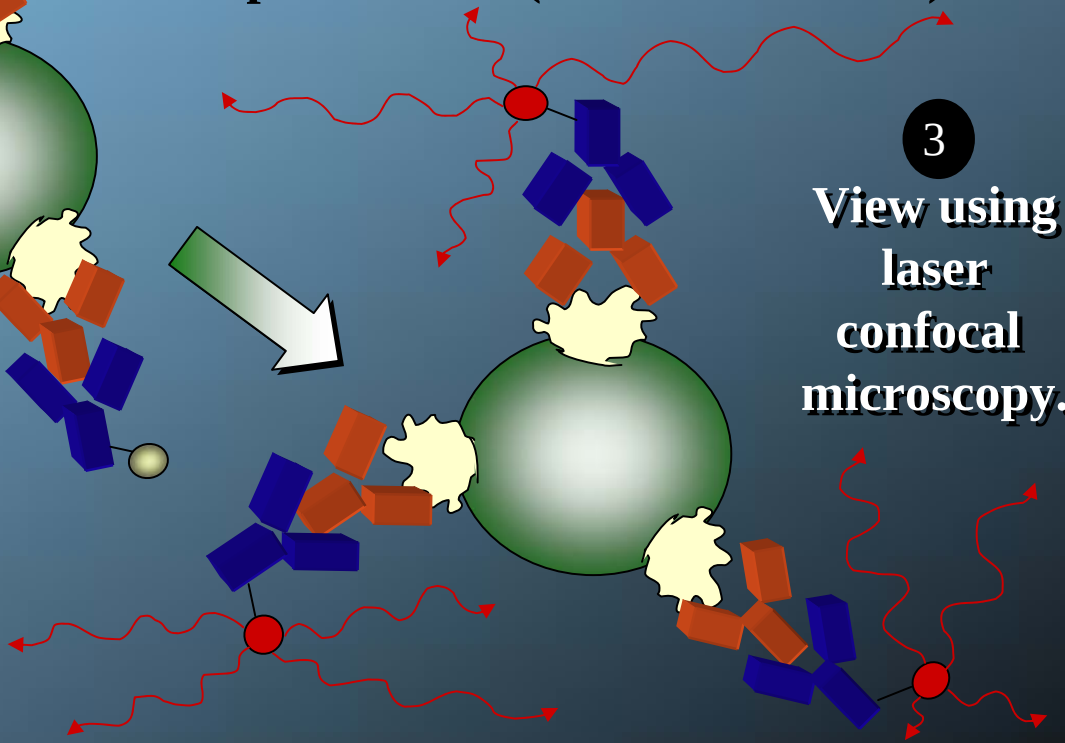
1

Prep intact embryos.



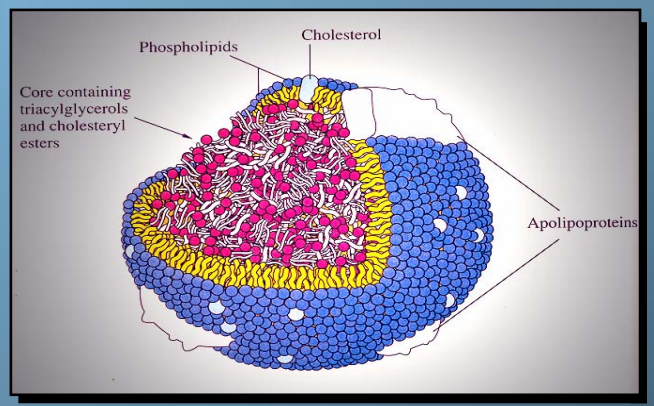
2

Incubate embryos in anti-vitellin antibody, secondary antibody, and neutral lipids-specific stain (BODIPY 505/515).

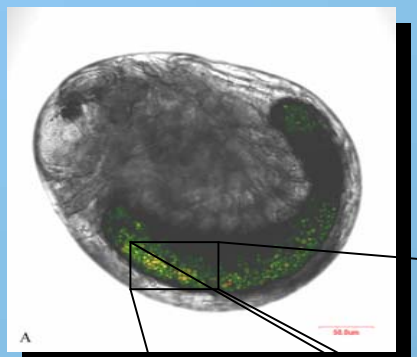


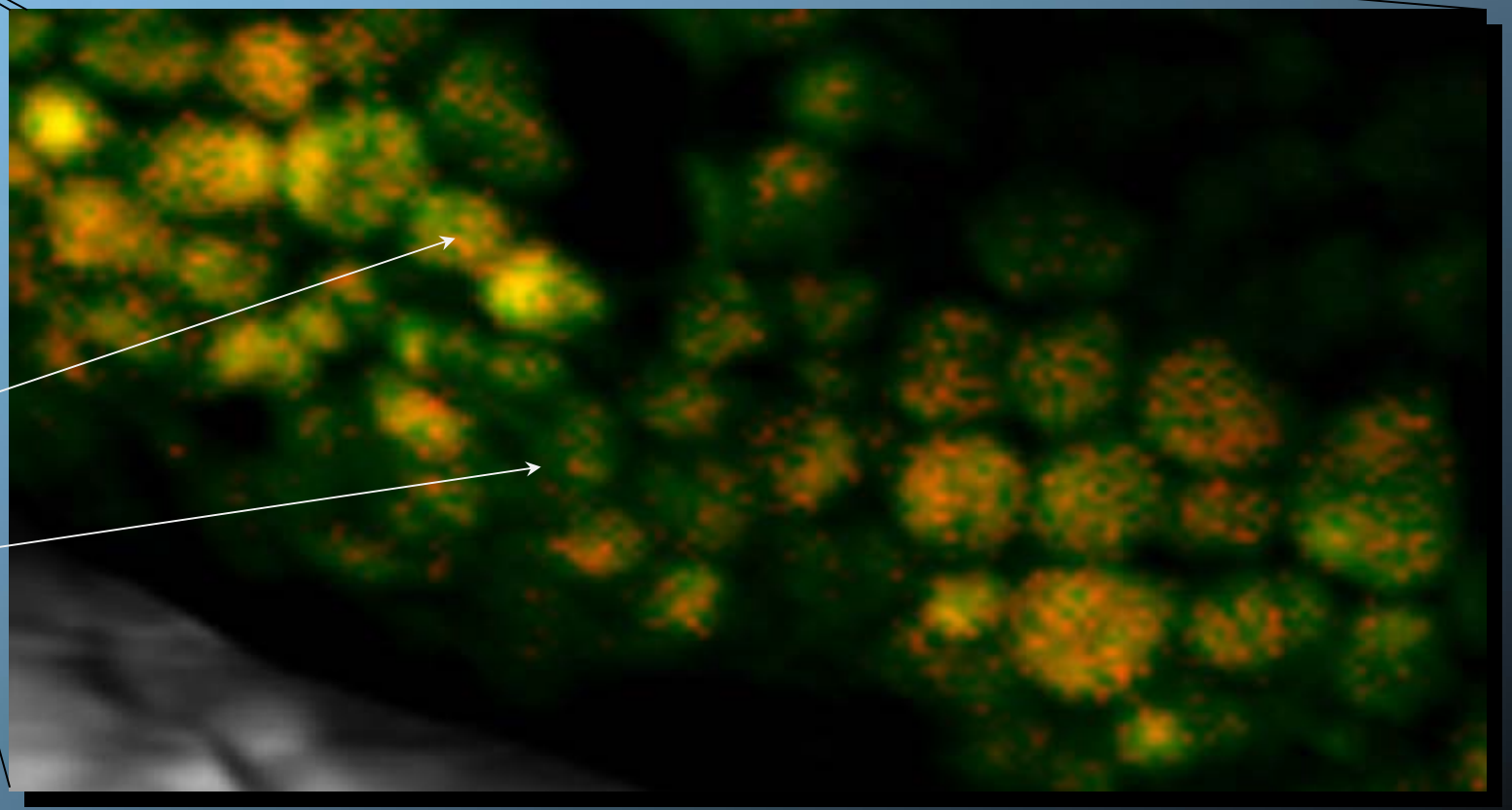
3

View using laser confocal microscopy.



High Magnification of Yolk Granules

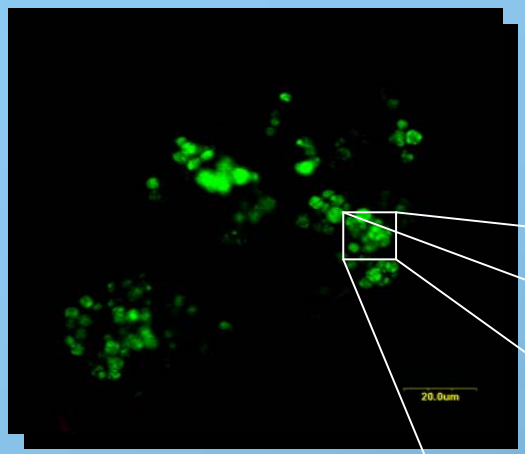


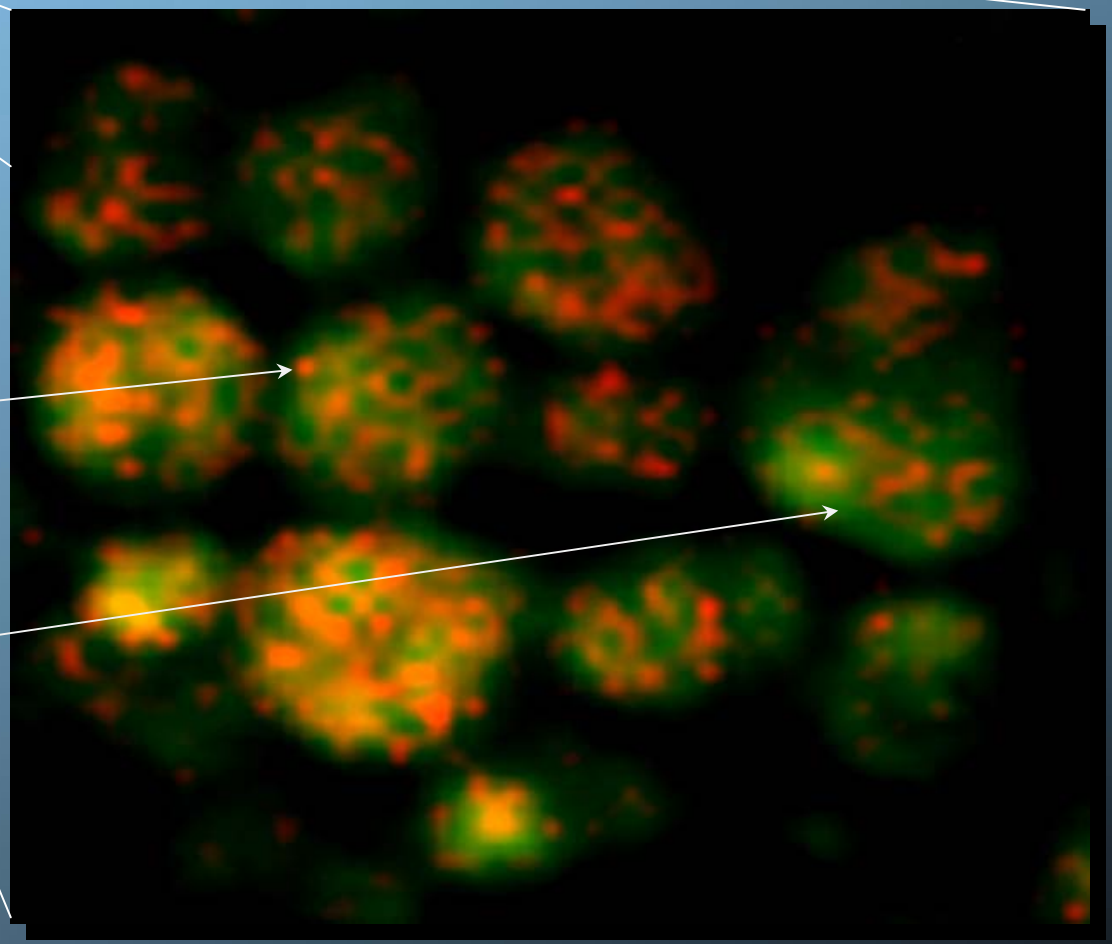


Vitellin Protein

Neutral Lipids

High Magnification of Yolk Granules in Copepods





Vitellin Protein

Neutral Lipids

Applications of copepod molecular endpoints to assay EDC effects

- Lipovitellin titers in males can be useful biomarkers of feminization by EDCs acting through EcR/USP-mediated mechanisms.
- Lipovitellin titers in females (and eggs) can be useful as a surrogate measure of egg quality and production potential.
- Ecdysone (20HE) balance is dynamic, but may reveal EDC effects if assayed in multiple copepods over multiple lifecycle stages in microplate tests or sediment bioassays.
- Both biomarker systems can be applied to field populations since meiobenthic copepods are found almost everywhere in marine sediments.

Conclusions

- Receptor affinity extraction methods based on recombinant nuclear hormone receptors are being developed as bioanalytical tools to assay EDC exposure in complex mixtures
- These methods will be applied in combination with high performance mass spectrometric techniques to characterize EDCs in novel wastewater-impacted coastal estuarine systems
- Linkage of EDC exposure to biological effects is being pursued through vertebrate (zebrafish) and invertebrate (copepod) assays sensitive to receptor-mediated EDC mode of action

Acknowledgement