Acute plasticizer exposure to *Montipora capitata* egg-sperm bundle crosses

Project Overview

Plasticizers are a suite of chemicals added to plastic consumer products to increase flexibily. The most widely used plasticizers are phthalate acid esters (PAEs).

I exposed coral embryos and larvae to phthalates in filtered seawater and assess any toxic effects on the survival and development of the coral embryos. This experiment will be conducted in closed-system 20mL scintillation vials with foil-lined caps.

Materials

- 1mL of EPA 506 phthalate mix , 500μg/mL in methanol (keep refrigerated)

Glassware

- 6L of 1-micron filtered seawater (fsw), in glass jars baked at 500˚F (260˚C) for 1hr
- 6, 1L reagent bottle for stocks, baked at 500˚F (260˚C) for 1hr
- 1, 1000mL graduated cylinder for measuring out fsw

Pipettes & Tips

- 1, stereological 5mL pipette
- 6, stereological 5mL pipette tips
    - 1 for ea. concentration of phthalate
- 1, stereological 25mL pipette
- 6, stereological 25mL pipette tips
    - 1 for ea. concentration of phthalate
- 1, 1mL pipette and tips
- 1, 20-200μL p200 pipette and tips
- 1, 1-20μL p10 pipette and tips

PPE

- safety glasses (wear 'em!)
- nitrile gloves
    - From correspondence with UW EH&S Occupational Safety and Health practitioner Ann Tu:
        - "NIOSH has a PPE guide: https://www.osha.gov/sites/default/files/publications/osha3151.pdf and I only see two types of phthalates listed, "dibutyl phthalate" and "diocytl phthalate" on page 26 (printed on book) of the guide and looks like **nitrile** gloves would work for both. "

- Chemical hood (ventilation ON!)
- If you do not have access to a fume hood, use a respirator with P95 cartridges
    - From correspondence with UW EH&S Occupational Safety and Health practitioner Ann Tu:
        - "I used the 3M Respirator selection guide to look for the appropriate mask to protect against inhalation of phthalates https://multimedia.3m.com/mws/media/639110O/3m-respirator-selection-guide.pdf?fn=Respirator%20Selection%20Guide%20Final. Looks like **P95** would work for all the phthalates listed in the guide which includes all the above except benzyl butyl phthalate and di-n-octyl (they have di-sec-octyl which is the same as bis(2-ethylhexyl) phthalate."

Safety Considerations

Please review the SDS for EPA 506 phthalate mix, 500μg/L in methanol before working with this chemical!

Hazard statements:

- H225 Highly flammable liquid and vapor
- Toxic if swallowed, in contact with skin or if inhaled
- Causes damage to organs (Eyes, Central nervous system)

This chemical is toxic and can absorb through skin, and vaporize and be inhaled. **WEAR GLOVES, SAFETY GLASSES, & MASK. WORK UNDER A HOOD!** *If you are pregnant, consider alternatives like preparing leachate similar to Tetu et. al 2020*

Phthalates

I used the EPA 506 phthalate esters mix 1, which comes as a 1mL ampule vial with a glass break-off cap. It includes 6 common phthalates each at a concentration of 500μg / mL, *(500,000μg/L)* .

The mix contained the following 6 phthalates:

- Benzyl butyl phthalate **(BBP)**
- Bis(2-ethylhexyl) adipate **(DEHA)**
- Bis(2-ethylhexyl) phthalate **(DEHP)**
    - listed on EPA's Toxics Release Inventory (TRI)
    - DEHP is regulated under the Safe Drinking Water Act. The highest concentration allowed, the maximum contaminant level (MCL), is 0.006 mg/L , **(6μg/L)**
- Dibutyl pthalate **(DBP)**
    - listed on EPA's Toxics Release Inventory (TRI)
- Diethyl phthalate **(DEP)**
- Dimethyl phthalate **(DMP)**

Concentrations of phthalates in environmental sea water samples reported in the literature:

Study Area	BBP	DEHA	DEHP	DBP	DEP	DMP	$\sum$ PAE	Reference
Review							0.5 - 10 μ g/L	Lynch et al 2022
Tunisia			<LOD-168μ g/L	<LOD-30.5μ g/L	<LOD-17.0μ g/L			Jebara et al 2021
Mediterranean Coastal Spain		0.0021-0.304μ g/L	0.031-0.617 μg/L		0.024-0.483 μg/L	0.0028 - 0.142 μg/L		Sanchez-Avila et al 2012

[$\sum$**[PAE] range: 0.5 μg/L - 10 μg/L**]

Lynch, Jennifer M., Katrina Knauer, and Katherine R. Shaw. 2022. "Plastic Additives in the Ocean." In *Plastics and the Ocean*, edited by Anthony L. Andrady, 1st ed., 43–76. Wiley. https://doi.org/10.1002/9781119768432.ch2.

[**DEHP, range: <MDL-168μg/L, mean: 71.1μg/L, median: 45.7μg/L, detected in 92.7% of 165 water samples**]

Jebara, Amel, Ambrogina Albergamo, Rossana Rando, Angela Giorgia Potortì, Vincenzo Lo Turco, Hedi Ben Mansour, and Giuseppa Di Bella. 2021. "Phthalates and Non-Phthalate Plasticizers in Tunisian Marine Samples: Occurrence, Spatial Distribution and Seasonal Variation." *Marine Pollution Bulletin* 163 (February): 111967. https://doi.org/10.1016/j.marpolbul.2021.111967.

Sánchez-Avila, Juan, Romà Tauler, and Silvia Lacorte. 2012. "Organic Micropollutants in Coastal Waters from NW Mediterranean Sea: Sources Distribution and Potential Risk." *Environment International* 46 (October): 50–62. https://doi.org/10.1016/j.envint.2012.04.013.

Based on this review, I chose to doses at the following concentrations:

- 0μg/L : control
- 0.5μg/L (0.0005μg/mL): environmentally relevant value
- 5μg/L (0.005μg/mL) : would be considered just under acceptable drinking water
- 50μg/L (0.05μg/mL) : increasing by factor of 10
- 150μg/L (0.150μg/mL): max environmental relevance (Jebara et al. 2021, in Tunisia @ 168μ g/L)
- 500μg/L (0.500μg/mL): an overdose above environmental relevance to be used as a positive control

**Doses may be graphed best on a log scale x axis

Dilution Plan

Concentration 1 multiplied by volume 1 is equal to concentration 2 multiplied by volume 2.

$$C_1V_1 = C_2V_2$$

The EPA standard mix is 500μg/mL (or also 500,000μg/L). This is our known concentration. We also know our desired diluted concentration, and the volume we need. Here we solve for V_1, to find out how much of C_1 we need to add to filtered seawater make the total end volume V_2.

Prepare phthalate stock solutions in 1L reagent bottles

Final volume of stock solutions must be enough to use in subsequent serial dilutions and in sample vials over the course of three nights of spawning. Stock solutions must be kept between -3C to -8C in a chemical storage refrigerator (same as the EPA standards mix).

[ ] OVERDOSE: Make 500mL of 500μg/L using EPA standard mix

$$500\mu g(V_1) = 0.500\mu g(500mL)$$
$$V_1 = \frac{0.500\mu g(500mL)}{500\mu g}$$
$$V_1 = 0.5mL$$

$500mL - 0.5mL = 499.5mL$: volume of filtered seawater to add to 1000mL reagent jar *(Do this via graduated cylinder, then add via p200 pipette)*

$0.5mL = 500\mu L$: volume of EPA standard mix to add to 1000mL reagent jar to attain end volume of 500mL *(use p200 pipette)*

[ ] PEAK: Make 1000mL of 150μg/L using EPA standard mix

$$500\mu g(V_1) = 0.150\mu g(1000mL)$$
$$V_1 = \frac{0.150\mu g(1000mL)}{500\mu g}$$
$$V_1 = 0.3mL$$

$1000mL - 0.3mL = 999.7mL$: volume of filtered seawater to add to 1000mL reagent jar *(Do this via graduated cylinder, then add via p200 pipette)*

$0.3mL = 300\mu L$: volume of EPA standard mix to add to 1000mL reagent jar to attain end volume of 1000mL *(use p200 pipette)*

[ ] HIGH: Make 1000mL of 50μg/L stock using EPA standard mix

$$500\mu g(V_1) = 0.05\mu g(1000mL)$$
$$V_1 = \frac{0.05\mu g(1000mL)}{500\mu g}$$
$$V_1 = 0.1mL$$

$1000mL - 0.1mL = 999.9mL$: volume of filtered seawater to add to 1000mL reagent jar to (Do this via graduated cylinder, then add via stereological and p200 pipette)

$0.1mL = 100\mu L$: volume of EPA standard mix to add to 1000mL reagent jar to attain end volume of 1000mL *(use p200 pipette)*

[ ] MID: Make 1000mL of 5μg/L stock using 50μg/L stock

$$0.05\mu g(V_1) = 0.005\mu g(1000mL)$$
$$V_1 = \frac{0.005\mu g(1000mL)}{0.05\mu g}$$
$$V_1 = 100mL$$

$1000mL - 100mL = 900mL$: volume of filtered seawater to add to 1000mL reagent jar *(Do this via graduated cylinder)*

$100mL$: volume of 50μg/L stock to add to 0.005μg/mL stock 1000mL reagent jar to attain end volume of 1000mL *(do this via 4 pulls of 25mL serological pipette)*

[ ] LOW: Make 1000mL of 0.5μg/L stock using 5μg/L stock

$$0.005\mu g(V_1) = 0.0005\mu g(1000mL)$$
$$V_1 = \frac{0.0005\mu g(1000mL)}{0.005\mu g}$$
$$V_1 = 100mL$$

$1000mL - 100mL = 900mL$: volume of filtered seawater to add to 1000mL reagent jar *(Do this via graduated cylinder)*

$100mL$: volume of 5μg/L stock to add to 0.05μg/mL stock 1000mL reagent jar to attain end volume of 1000mL *(do this via 4 pulls of 25mL serological pipette)*

Prepare treatment 20mL scintillation vials

Using a 20mL serological pipette, pipette 19mL from the desired stock concentration into the 20mL scintillation vial. Label vial lids with treatment concentration.

Collect egg-sperm bundles and perform bundle-bundle crosses

On spawning night, collect genetically disparate bundles and cross them (2 bundles per vial)

Count cleavage percentage 4-hours post fertilization

Set vials floating in a flow-through water bath, using diced pool floats. Let sit for 4 hours. After 4 hours, eggs should be broken up from the bundles and floating at the surface of the scintillation vial. Use a transfer pipette to move the eggs from the vial to a petri dish, and photograph all eggs from each treatment vial through an Amscope connected to a dissecting microscope.

Make sure to accurately name each photo jpeg file to reflect the treatment it came from. Work quickly and randomize the treatments you photograph. This part is very time consuming and will take 10 or more minutes per vial until you get the hang of it and start to work a bit faster (4-5 minutes per vial).