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## Internalization, reduced growth, and behavioral effects following exposure to micro and nano tire particles in two estuarine indicator species

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### Abstract

Synthetic rubber emissions from automobile tires are common in aquatic ecosystems. To assess potential impacts on exposed organisms, early life stages of the estuarine indicator species Inland Silverside (*Menidia beryllina*) and mysid shrimp (*Americamysis bahia*) were exposed to three

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tire particle (TP) concentrations at micro and nano size fractions (0.0038, 0.0378 and 3.778 mg/L in mass concentrations for micro size particles), and separately to leachate, across a 5-25 PSU salinity gradient. Following exposure, *M. beryllina* and *A. bahia* had significantly altered swimming behaviors, such as increased freezing, changes in positioning, and total distance moved, which could lead to an increased risk of predation and foraging challenges in the wild. Growth for both *A. bahia* and *M. beryllina* was reduced in a concentration-dependent manner when exposed to micro-TP, whereas *M. beryllina* also demonstrated reduced growth when exposed to nano-TP (except lowest concentration). TP internalization was dependent on the exposure salinity in both taxa. The presence of adverse effects in *M. beryllina* and *A. bahia* indicate that even at current environmental levels of tire-related pollution, which are expected to continue to increase, aquatic ecosystems may be experiencing negative impacts.

## Keywords

Tires; behavior; growth; salinity

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## 1. Introduction

An estimated 4.4 – 12.7 million metric tons of marine debris enter the ocean annually, presenting a threat to pelagic, benthic, and coastal environments (Granek et al., 2020; Jambeck et al., 2015; Rochman et al., 2016). Through photodegradation and weathering processes, these synthetic polymers fragment and are dispersed throughout the ocean, often concentrating in coastal areas (Barnes et al., 2009). Synthetic rubber emissions from automobile tires, now broadly considered a common type of microplastic (CA Waterboard, 2020), are a likely threat to the health of marine ecosystems, especially in estuaries, rivers, and streams located near metropolitan areas and busy roadways (Brahney et al., 2021; Gray et al., 2018; Klöckner et al., 2019; Rochman et al., 2019; Tian et al., 2021; Wagner et al., 2018). Another potential pathway of tire particles (TP going forward) is stormwater runoff, as reported by a recent study at 12 sites within the San Francisco Bay estuary, where fibers and TP (black rubbery fragments) contributed ~85% of total particles sampled (Werbowski et al., 2021). Modern tire materials, products of fossil fuels, are composed of complex mixtures of synthetic polymers, natural rubbers, carbon black, polyester and nylon fiber, chemical additives, petroleum, and pigments (Baumann and Ismeier, 1998). These mixtures are shed as TP, characterized as airborne and road wear particles, generated by the rolling shear of tread against a surface (Kovochich et al., 2021; Rogge et al., 1993). Once produced, TP can aggregate with other auto-related particles from brake dust, pavement, and atmospheric deposition (Charters et al., 2015). The presence of these particles in aquatic environments may result in impacts to wildlife and humans. For example, changes in cell morphology and DNA damage due to inhalation of tire particles are known to occur in humans (Gualtieri et al., 2008). Additionally, tires can leach constituents known to be toxic to aquatic organisms across different taxonomic orders (Hartwell et al., 2000; Nelson et al., 1994; Tian et al., 2021). For example, TP has been recently documented to cause acute toxicity to Coho salmon due to the presence of 6PPD-quinone, a chemical commonly used as an antiozonant and antioxidant in tires. (Tian et al., 2021).

Coastal estuaries are susceptible to micro and nano plastic pollution from terrestrial sources, including automobile tires. These water bodies receive freshwater from inland rivers, which deliver nutrients and runoff that may harbor agricultural chemicals and microplastics (Le Roux, 2005). An automobile tire is designed to last for 40,000 km until it is worn down, and throughout its lifetime, about 30% of its tread erodes and enters the environment (Dannis, 1974; Piotrowska et al., 2019). It is estimated that coastal rivers in Europe transport an annual load of 1.2 kt of TP to the Atlantic Ocean (Siegfried et al., 2017). Knowledge on the distribution and concentration (mass or particle count) of TP in coastal areas is limited (Unice et al., 2019). In Charleston Harbor, TP was found in all layers (intertidal sediment, subtidal sediment, and sea surface micro layer) with a maximum concentration identified in the intertidal sediments of the Ashley River (203 mg/Kg ww) (Gray et al., 2018). Another study report predicted average coastal European surface water concentrations to contain 0.03-17.9 mg/L and measured 0.09-6.4 mg/L of TP (Wik and Dave, 2009), which is in range the mass concentration used in this study (0.0038-3.778 mg/L ww).

Once in an estuary, low-density microplastics, including TP, remain buoyant for a period of time and become available to planktonic organisms which may ingest these fragmented particles (Barnes et al., 2009). As predators consume prey organisms, those particles are susceptible to trophic transfer in estuarine food webs (Athey et al., 2020; Au et al., 2017; Stienbarger et al., 2021). Several recent studies have indicated that estuarine species such as shore crabs, oysters, shrimp, fish, and clams will internalize microplastics through ingestion and uptake through gill tissue and soft tissues (Bessa et al., 2018; Davidson and Dudas, 2016; Gray and Weinstein, 2017; Van Cauwenberghe and Janssen, 2014; Watts et al., 2014). At the same time, organisms inhabiting estuaries are exposed to a wide range of salinities, which may alter the impacts of pollutants as freshwater transitions to saltwater, and in terms of micro and nanoplastics may influence agglomeration and hence bioavailability (Shupe et al. 2021). Testing across salinities is important because as global ocean temperatures warm, salinity is evidenced to increase (Durack et al., 2012; Helm et al., 2010). This increase in salinity may alter or potentiate the effects of pollutants, including micro and nanoplastics (MNPs), on estuarine organisms (Hutton et al., 2021; Shupe et al., 2021).

*Americamysis bahia* and *Menidia beryllina* are model estuarine organisms used across a range of salinities following guidelines developed by the EPA for whole effluent toxicity testing (Brander et al., 2012; Pillard et al., 1999; Vlaming et al., 2000). Changes in organism behavior result from various cellular, biochemical, and neural processes (Døving, 1991; Little, 1990) that are critical to organism survival as well as fitness, thus a sensitive endpoint for use in toxicity testing (USEPA, 1994). Numerous studies have drawn links between the biogeochemical and ecological consequences of environmental contamination by demonstrating that subtle changes in fish behavior indicate stress (Beitinger, 1990; Little, 1990; Sprague, 1971). Swimming and feeding behavior, frequency of activity, and velocity have been established as reliable responses to measure sublethal toxicity stress in fish (Grillitsch et al., 1999; Little and Finger, 1990; Newman and Jagoe, 1996) and that also has implications for organism fitness (Weis et al., 2001). The current experiment synthesizes methods of early and recent studies to measure several of these historically documented stress responses as well as growth in *M. beryllina* and *A. bahia* using periodic light and dark cycles as introduced stimuli (Pannetier et al., 2020; Romney et al., 2019). The purposes

of the light/ dark cycles are to provide a general overview of organismal behavior in the environment during these conditions, as well as a stimulus effect for fish to act on, as in the natural environment.

This study investigated the sublethal effects (behavior and growth) of micro (10-20  $\mu\text{m}$ ) and nano ( $< 1\mu\text{m}$ ) TP exposure across a salinity gradient similar to that found in estuaries. Subtle changes in behavior and growth are essential to document because they may increase predation risk and population-level effects (Beitinger, 1990; Little and Finger, 1990; Mundy et al., 2020). We used a range of concentrations based on environmentally relevant mass concentrations of TP and its leachate on behavior in the early life stages of indicator species *A. bahia* and *M. beryllina*. We hypothesize that TP will influence both growth and behavior, that it will be readily internalized, as has been demonstrated across other microplastic types in the early life stages of aquatic organisms, and that some responses may be salinity and size dependent. As data on TP pollution and sub-lethal effects in aquatic species are currently rare, this study fills critical knowledge gaps on uptake and internalization, growth impacts, and stress responses to an emerging microplastic pollutant by species that may act as proxies for threatened or endangered species and ecosystems sensitive to anthropogenic pollution.

## 2. Methods

### 2.1 Chemicals

Suwanee River Natural organic matter (NOM) - 2R101N used to create suspensions of MNPs in exposure wells was purchased from the International Humic Substance Society, St. Paul, MN. Tissue-Clearing Reagent CUBIC-R+ [for Animals] (T3741) and Tissue-Clearing Reagent CUBIC-L [for Animals] (T3740) for visualization of particles within organisms following exposures were purchased from Tokyo Chemical Industry Co., Ltd.

### 2.2 Microplastics preparation

A detailed TP preparation protocol has been provided in SI. Briefly, TP from tire tread was prepared by cryomill process in a ceramic chamber (Retsch CryoMill, Haan, Germany). After milling, 3 g tire particles were combined with 300 ml of solution in a flask containing 50 mg/L Suwanee River NOM prepared in Milli-Q water then filtered through a 0.2 mm filter. The solution is then run through a coarse strainer to remove the glass beads and strained through a 20  $\mu\text{m}$  standard mesh sieve, producing a resulting solution with particles  $<20\ \mu\text{m}$  in at least one dimension. Then, using a 47 mm syringe filter holder containing a 1  $\mu\text{m}$  mixed cellulose ester (Advantec) filter the solution is further filtered to produce a suspension of nanoparticles  $<1\ \mu\text{m}$  in at least one dimension. The filter holder is then backflushed with clean NOM suspension, and the backflushed solution is collected to produce a suspension of tire particles in the range of 1-20  $\mu\text{m}$ . A portion of the prepared nano-TP fraction was further filtered using a 30K MWCO centrifugal filter (Corning Spin-X #431489) ran at 7800 rpm for 5 minutes to rinse particles and to produce simulated TP leachate. The solution particle counts are determined separately for each fraction of the suspension. The micron (1-20  $\mu\text{m}$ ) sample particle count is determined by triplicate sampling of the suspension and the particle count analysis by flow cytometry

(Acurri C6 Flow Cytometer, BD Biosciences, San Jose, CA). The nanoscale (<1  $\mu\text{m}$ ) sample particle count is also determined in triplicate by Nanoparticle Tracking Analysis (NTA) on a NanoSight instrument (NanoSight NS500, Malvern Instruments, Westborough, MA).

### 2.3 Model organisms, their sources, and experimental setup

*Americamysis bahia* larvae were purchased from Aquatic Biosystems in Fort Collins, Colorado and reared in three tanks at 15, 20, and 25 PSU salinities with filtered artificial seawater prepared (AFSW). For each organism, there were three biological replicates. For silversides, 2 technical replicates were averaged for each of the three biological replicates. For mysids, 3 technical replicates were averaged for each of the three biological replicates. Following EPA protocol 833-C-09-001 (USEPA, 2009), when adult *A. bahia* reproduced, larvae were moved to additional tanks of the same salinity and reared for seven days prior to exposures beginning. Micro and nano-TP exposures with mysids were initiated at seven days post fertilization (dpf) (n=3) under static renewal conditions for seven days. *Menidia beryllina* embryos were harvested from broodstock held at the Hatfield Marine Science Center into three acclimation aquaria of 5, 15, and 25 PSU salinities with filtered AFSW following modified methods from Middaugh et al. (1987) as done in previous studies in the Brander lab (e.g. DeCourten et al., 2020; Hutton et al., 2021). Larvae were placed into exposure vessels at  $6 \pm 1$  days post fertilization (dpf) (n=6 technical replicates to make n=3 biological replicates) and maintained under static renewal conditions for 96 h. All exposure vessels were covered during exposures to prevent background contamination and a blank filter water was also used.

Each model species was exposed to a total of 26 treatments (n=3): each containing a water control, NOM control with four TP concentration treatments (micro and nano with 60, 6000, and 60000 particles/mL, which is equivalent to 0.0038, 0.378 and 3.778 mg/L in mass concentration for micro-size particles; 0.014% TP leachate) across three salinities per species as described above. Nominal water concentrations with detailed QA/QC are provided in SI table 1. Water quality parameters were measured daily over the exposure period at the time of 80% water renewal. Cumulative hatching and mortality were recorded daily. *A. bahia* were fed concentrated brine shrimp (*Artemia franciscana*) ad libitum, and *M. beryllina* were fed Gemma Microdiet 0.2 mg/beaker/day (Skretting, Westbrook, Maine). Both organisms were fed daily and allowed to feed for at least two hours before water changed. Table SI 2 and 3 provides water quality parameters maintained throughout the experiment. A control blank filters were setup in a petri dish to measure background contamination. No particles resembling TP were observed on filter blanks.

### 2.4 Behavioral assays

Following MNP exposures of 7d (*A. bahia*) and 96 h (*M. beryllina*), behavioral assays were performed post-exposure from each treatment using a DanioVision Observation Chamber (Noldus, Wageningen, the Netherlands) for the dark: light cycle as described previously (Mundy et al., 2021; Segarra et al., 2021). Briefly, *A. bahia* and *M. beryllina* larvae were randomized and placed in individual 10 ml glass beakers within a 12-well plates tray designed and 3D-printed in Brander lab (Hutton et al., 2021), in the Ethovision Observation Chamber (EOB) to observe natural photo motor response. Larvae were acclimatized for

at least 1 hour before placing into the EOB. After acclimatization outside the chamber, another 5-minute acclimatization period was provided inside the dark chamber, followed by three cycles of alternating 2-minute intervals of dark stimuli and 2-minute intervals of light stimuli. Behavior and activity were recorded and tracked by a Basler Gen 1 Camera using Ethovision XT15 software. Velocity thresholds were determined for swimming parameters between 0.5 cm/s (freezing) – 2.0 cm/s (moving) (Segarra et al., 2021). A virtual center zone (1.6 cm diameter) was established to measure the time that larvae spent in the center of the 2.2 cm diameter in the beaker. All behavioral tests were conducted between 09:00 and 18:00 h. The resolution was set at 1280 x 960, light cycles were programmed at 10,000 lux and the frame rate was set at 25/s. A total of seven variables were analyzed in this study which is included in Table 1. Following behavioral analysis, organisms were euthanized humanely, silversides per IACUC protocol #0035, and fixed in paraformaldehyde (PFA) to preserve tissues for examination of MP internalization.

## 2.5 Growth and TP internalization

At least three individuals from each species per treatment were collected for growth measurement. Length and width measurements were collected via dissecting scope equipped with Moticam visual software, and particle uptake was visualized on a Zeiss Axio Observer inverted microscope (Carl Zeiss, White Plains, NY). Growth data were assessed by creating a growth index with the following formula:

$$\frac{W}{L}Xd$$

Where W is the width of the organism, L is the length, and d is the number of days the organism is exposed to the TP. This relationship provides the index used to plot the final growth curve. Organisms were then cleared using a protocol adapted for larval organisms with CUBIC™ clearing reagents (Ohnuma et al., 2017; Susaki et al., 2015). Briefly, to remove pigmentation and allow visualization of internalized microplastics (1-20 µm), individual organisms fixed in 3% PFA were washed in 5 ml phosphate-buffered saline (PBS) for 30 minutes and incubated in 5 ml CUBIC-L at 37 ° C for seven days to encourage lipid removal. Following this step, organisms were washed again in 5 ml PBS for an additional two hours and then transferred to CUBIC-R + for an additional seven days to clear the remaining tissue.

## 2.6 Statistical analysis

Statistical analysis was performed using RStudio Version 1.0.153. Dose-response curves were generated to evaluate larval swimming behavior and growth effects across concentration treatments. The growth data were analyzed using a maximum likelihood estimate (MLE) approach to evaluate which of five different curves (linear regression, quadratic, sigmoidal, 5-parameter unimodal, and 6-parameter unimodal) were tested for the best fit to all three concentrations and controls. A maximum likelihood ratio test was used to examine whether each curve provided a better fit than an intercept-only null model with a significance level of  $\alpha < 0.05$ . All calculations for the concentration–effect curves were performed using mean behavior variables, re-scaled between 0 and 1 within each cycle

to facilitate comparison between salinity. R scripts used for data preparation, statistical analysis, and graphing can be found at <https://github.com/branderlab/TWP-DRC-Curve.git>, and examples using the same package are published in other studies (Brander et al., 2016; Frank et al., 2019; Mundy et al., 2020) Concentration dependent dose response curves for behavioral data were prepared by `drm` function in R using DRC package by Ritz, (2010), which does not include leachate (due to the absence of particle count). The Shapiro–Wilk test was used to test normality, and Levene’s test was used for homogeneity testing. After confirming normality and homogeneity of data, a 3-4 parameter using nonlinear regression approach was used to prepare the model at each salinity and combined using `ggplot2` function in R. Analysis of Variance (ANOVA) was used to evaluate differences among treatment groups. A Tukey HSD post-hoc test was used to compare particle concentrations between treatments, and a Dunnett’s post-hoc test was used to compare leachate treatments to controls. Differences were considered statistically significant at  $p < 0.05$ .

### 3. Results and discussion

#### 3.1 Behavioral responses of model species when exposed to TP in a range of salinities

**3.1.1 Behavioral responses for *A. bahia***—Average *A. bahia* larvae survival for control and exposure treatments was  $98 \pm 2\%$  and  $90 \pm 3\%$ , respectively, with no significant difference across the treatments (ANOVA (Normal distribution, Tukey HSD post-hoc,  $p < 0.05$ )). Out of all seven behavioral responses analyzed, ~50% of micro- TP exposures and ~33% of nano-TP (except 25 psu; ~57%) exposures were significantly different from the control group in at least one concentration in both light and dark cycle at least one salinity (SI Table 4; Fig. SI Fig. 1C & D). In both the micro and nano-sized TP treatments, *A. bahia* turn angle, freezing, movement and in zone duration (time spent in center of beaker) were most significantly affected at each salinity (Fig.1). In leachate-exposed *A. bahia*, six out of the seven variables (freezing, movement, In zone duration, frequency, meander and turn angle) were significantly different from the control group (SI Fig. 1B).

When compared between dark and light cycles, *A. bahia* demonstrated increased distance and meander in the light cycle at highest salinity, with increasing freezing frequency and time spent in the zone at lowest salinity in micro and nano TP exposed group (Fig. 1 & 3). When compared between TP sizes, nano-TP caused hyperactivity in *A. bahia*, reflected by their swimming distances significantly increasing in a concentration dependent manner. Selected variables (distance, in zone duration, meander and turn angle) in the dark and light cycle micro-TP demonstrated about 70% behavioral alterations compared to control whereas in nano-TP exposure group about 80% behavioral alterations in both dark and light cycles at at least one salinity (SI Table 4; SI Fig. 1).

In terms of salinity, behavioral alterations in both nano and micro-TP exposures were significantly higher at the two higher salinities (15 and 25 PSU). This suggests nano-TP affected mysids more at a higher salinity as reported by other studies (Kögel et al., 2020; Lee et al., 2013; Rist et al., 2017). This may be due to agglomeration at higher salinities (Shupe et al. 2021). When *D. magna* were exposed to nano (1-9  $\mu\text{m}$ ) and micro (>10  $\mu\text{m}$ ) plastics, nanoplastic was reported to decrease 21% feeding rates compared to microplastic exposure (Rist et al., 2017). Other studies reported hyperactive behavior in zebrafish

(*Danio rerio*) exposed to micro polystyrene (PS) (0.001-20 mg/L, equals to  $14.5\sim 2.9 \times 10^5$  particles/mL) and sticklebacks (*Gasterosteus aculeatus*) exposed to PE (50,000 particles/ml) each, (Bour et al., 2020; Chen et al., 2020). Moreover, hyperactivity has been reported in the F1 offspring of zebrafish exposed to polyvinyl chloride (PVC) and high-density polyethylene (HDPE) (Cormier, 2020).

**3.1.2 Behavioral responses for *M. beryllina***—Average *M. beryllina* larvae survival for control and exposure treatments was  $97 \pm 3\%$  and  $91 \pm 2\%$ , respectively, with no significant difference across the treatments (Normal distribution, Tukey HSD post-hoc,  $p < 0.05$ ). In *M. beryllina*, all the behavioral variables measured were significantly differently from the control group in at least one salinity (SI Table. 5; SI Fig. 2C & D). In both dark and light cycles, *M. beryllina* spent an increased time in the zone (the center of the beaker) compared to controls, and also had an increased turn angle (Fig. 2 & 4). Dark and light cycle behavioral observations showed a similar pattern except *M. beryllina* meandered more compared to the dark cycle at all the exposure concentrations, within at least one salinity condition. Following TP leachate exposure concentration, more than half of the variables (freezing, movement, In zone duration, frequency, meander and turn angle) demonstrated a significant change in behavior from the control in both dark and light cycles in at least one salinity condition (SI Table 5; SI Fig. 2B). Similar behavior changes were observed in Delta smelt (*Hypomesus transpacificus*) following exposure to pesticides (Mundy et al., 2021, 2020). Concentration dependent dose response curves for the selective variables demonstrated 79% behavioral alterations in micro-TP exposed silversides whereas, in nano-TP exposure group, about 75% behavioral alterations at both micro and nano exposed TP Silversides at both dark and light cycle (Fig. 4). In silversides salinity dependent behavioral changes were not significant in the micro-TP exposed group, in contrast to nano-TP where higher salinity seems to affect behavior more. This may be because of increased agglomeration of nano-TP at higher salinities (Shupe et al. 2021; Gousiadou et al., 2021), as this response to nano TP at higher salinities was also seen in mysids. Altered swimming behavior, reduced velocity and decreased feeding activity have also been observed in larval zebrafish (*Danio rerio*), larval rockfish (*Sebastes schlegelii*) and sheepshead minnow (*Cyprinodon variegatus*) when exposed to PS and polyethylene (PE) microplastics (Chen et al., 2017; Choi et al., 2018; Noldus et al., 2001; Yin et al., 2019, 2018).

**3.1.3 Comparable Behavioral responses for *M. beryllina* and *A. bahia***—When comparing *M. beryllina* and *A. bahia* behavioral responses, there were some correlations (Pearson) identified between some variables (SI Table 6). There was a direct correlation observed in *M. beryllina* between distance related to movement, freezing (0.32-Dark, 0.16-Light) (Fig. 2). In contrast to mysid shrimp, there was an inverse relationship between movement and freezing ( $-0.54$ -Dark,  $-0.59$ -Light) (Fig. 1). Freezing demonstrated a weak inverse relationship with velocity for *M. beryllina* and *A. bahia*. Turn angle and freezing mean also showed a weak inverse relationship with the movement for *M. beryllina* and *A. bahia*. This suggests random movement that can be caused by additional stress due to the TP.



### 3.1.4 Salinity impacts on Behavioral responses for *M. beryllina* and *A. bahia*

—Within different salinities, the lowest salinity (15 PSU) in *A. bahia* demonstrated the highest variation from control in combined dark and light cycles in all concentrations of micro-TP (SI Table 4; Fig.1). This was in contrast to nano-TP and leachates, where the highest salinity (25 PSU) demonstrated the most impact on behavioral variation. As mentioned above, this may be due to agglomeration behavior, and also due to some soluble chemicals becoming more bioavailable at higher salinities, and thus potentially more toxic, although this requires further research (Hutton et al., 2021; Saranjampour et al., 2017). Similar results were observed in *M. beryllina*, where in both the dark and light cycle the lowest salinity (5 PSU) showed most variation from control when exposed to micro-TP in contrast to nano-TP and leachate exposure group, where the most behavioral variation from control was seen in highest salinity (25 PSU) (SI Table 5; Fig. 2). These results align with recent findings on nanoplastics agglomerating more as salinity increases (Shupe et al., 2021). *M. beryllina* exposed to both micro and nano-TP exhibited increased duration of time spent in central habitat across all concentrations and salinities, except individuals exposed to nano-TP in the highest concentration and TP leachate, both at lowest salinity in dark cycles. Similarly, *A. bahia* exhibited increased in zone duration across all TP concentrations, salinities, and light-dark cycles, including individuals exposed to TP leachate. Occupancy of the boundaries of a novel environment is widely documented to indicate a stress response in fish, rodents, and humans (Kallai et al., 2007; Schnörr et al., 2012; Sharma et al., 2009; Treit and Fundytus, 1988). An increase in central habitat occupancy that is significantly different from control organisms may indicate increased exploration or indiscriminate feeding behavior. Previous studies observed impaired swimming competence and reduced exploratory behavior in *N. japonica* exposed to PS microbeads (Wang et al., 2020). The uninhibited exploration behavior we observed may lead to an increased risk of predation in these highly susceptible larval fish.

Behavioral changes can be an outcome of physiological changes like respiratory stress (Abdel-Tawwab et al., 2019; Hashemi et al., 2019) that may be caused by changes in oxygen consumption with altered ion regulation (Kolandhasamy et al., 2018; Watts et al., 2016) as observed in this study, where increasing zone duration and freezing are caused at various TP concentrations (Fig. 1& 2. ; SI Table 4&5). Similarly, ingestion of irregularly sized TP may also induce irregular behavior (Wang et al., 2016; Wright et al., 2013) and may be another reason for irregular behavior patterns in our study (Fig 1 and 2). These particles can also come in contact with the skin, gills, fins, and eyes of the organisms, when present in high concentrations, and may result in abnormal swimming behavior (Choi et al., 2018), as observed in this study with altered turn angle and meandering patterns differing from control (Fig. 1&2; SI Table 4&5). Altered turn angle and meandering patterns describe necessary behavioral patterns required by an aquatic organism for their survival, supporting actions such as predator avoidance or foraging. Some of the behaviors documented in our study (in zone, turning, and velocity) may represent hyperactive behavior of an organisms (Mundy et al., 2021). Behaviors exhibited by organisms exposed to tire particles herein may also be indicative of exploration avoidance, or an indication of anxiety-like behavior (e.g., altered in zone duration, Schnörr et al., 2012). If an organism can't respond quickly to prey or a potential predator and gets confused due to the presence of external particles it may

limit their ability to survive, causing long term population decline (Weis and Candelmo, 2012). Several studies have reported that MNPs can cause movement-related neurotoxicity in organisms (Barboza et al., 2018; Lei et al., 2018; Yin et al., 2018), as reported in this study, with changing movement and distance in model species when exposed to various TP concentrations indicating neurotoxicity as other studies have with other polymer types. Swimming behavior is crucial for predator defense and avoidance, food acquisition, and social activity (Colwill and Creton, 2011) that all require motor as well as sensory systems (Roberts et al., 2011; Wong et al., 2010) to work in concert. *M. beryllina* are known to occur in schools and exhibit diel migrations following zooplankton prey, often displaying high school densities during the nocturnal period, presumably to reduce predation (Wurtsbaugh and Li, 1985). The presence of high TP concentrations may alter migration or shoaling patterns and limit population ranges, although environmentally relevant TP concentrations in larger water bodies may not present significant risk at this time. *A. bahia* exposed to nano-TP concentrations (60 and 6,000 p/ml) at 15 PSU and 25 PSU in dark cycles exhibited further total distance moved while organisms exposed to TP leachate in light cycles exhibited shorter total distance moved at 25 PSU salinity. Increased activity from TP exposure in nocturnal periods may not present high risk to the diurnally benthic *A. bahia*, which becomes planktonic at night to forage for food and engage in reproductive activity (Wortham-Neal and Price, 2002). However, observations of decreased activity resulting from TP leachate exposure in *A. bahia* during diurnal periods may increase susceptibility to predation by fish or crustaceans who forage during the day or cause reduced food intake.

### 3.2 Growth and Ingestion

*A. bahia* growth demonstrated a significant concentration-dependent decrease in both the highest salinities (Normal distribution, Post-hoc Tukey's test, ANOVA,  $p < 0.05$ ) micro-TP exposure (Fig. 5). There were no significant differences observed in leachate concentration when compared to control (Fig. 7A). When compared between the salinities, *A. bahia* demonstrated comparatively better growth at the highest salinity, which was reduced significantly at the highest TP concentration (Tukey HSD post-hoc, ANOVA,  $p < 0.05$ ). There was no significant growth reduction demonstrated in nano-TP-exposed *A. bahia* over all concentrations. However, the highest salinity demonstrated better growth compared to both lower salinities. The appearance of ingested TP was concentration-dependent in *A. bahia*, as shown in Fig. 8A and 9A. TP ingestion is also documented in other benthic invertebrates (Khan et al., 2019; Redondo-Hasselerharm et al., 2018). In the case of amphipod crustacean (*Hyallela azteca*), gut retention times of 24–48 h were observed in ingested TP with a significant impact on net growth when exposed to 500–2000 p/ml (Khan et al., 2019).

*M. beryllina* demonstrated significant concentration-dependent reduced growth in both micro- and nano-TP exposed groups at all salinities, except in nano-TP group at lowest salinity (Tukey HSD post-hoc ANOVA,  $p < 0.05$ ; Fig. 6). There were no significant differences observed in leachate concentration when compared to control (Fig. 7B). This is true in the case of *M. beryllina*'s ingestion of micro-TP as well, where ingested particles were observed at the two highest concentrations with the highest number of ingested TP at middle salinity (Fig. 7B and 8B). This is consistent with a recent study that traced TP in the

gut of 14% of individuals across five fish species surveyed in urbanized estuarine conditions (Parker et al., 2020).

Micro and nanoplastic exposures can cause adverse effects on the growth and development of larval aquatic organisms, primarily through ingestion (Athey et al., 2020; Lo and Chan, 2018). Inhibited growth may reduce the probability of attack because of inconspicuousness, but in the long term may increase failure to escape as a result of less developed sensory and locomotion abilities (Fuiman and Magurran, 1994). Further, reduced growth and stunted development increase the amount of time a larval organism spends in a specific stage or size class, impacting cumulative predation mortality rate (Shepherd and Cushing, 1980), and could also result in reduced size at reproductive maturity (e.g. DeCourten and Brander, 2017). In *M. beryllina*, optimal growth in laboratory conditions has been documented at 15 PSU while Mysid species was 30 PSU (Middaugh et al., 1987). Therefore, measuring growth in larval individuals following a period of salinity stress may yield unique insight into the effects of TP across different salinities on developing organisms. Particularly considering that salinity regimes are already being altered by global climate change (DeCourten et al., 2019).

Ingestion of 1 – 20 mm micro-TP was observed in *A. bahia* and *M. beryllina* at 6000 p/ml and 60,000 p/ml. A generalized linear model (GLM) was run for particle count at all three concentrations (micro-TP) at different salinities for both the model species (Fig. 8A and B). The GLM for *A. bahia* suggested concentration dependent ingestion at all the salinities ( $\chi^2 = 18.12$ ,  $df = 3$ ,  $p < 0.005$ ). Results also suggested concentration dependent ingestion at the lowest salinities (5 and 15 PSU) ( $\chi^2 = 2.55$ ,  $df = 3$ ,  $p < 0.005$ ). However, in *M. beryllina* in 15 PSU demonstrated increasing ingestion compared to the 5 and 25 PSU salinity group ( $\chi^2 = 1.04$ ,  $df = 6$ ,  $p < 0.005$ ). Results suggest that ingestion is likely the most common interaction that nondiscriminatory feeding fish larvae and zooplankton have with TP. Previous studies confirm that *M. beryllina* will ingest microplastic at high concentrations when exposed to zooplankton internalized with TP, although most particles were observed to be egested within 24 hours of internalization (Athey et al., 2020). However, the gut retention time for TP in *A. bahia* is unknown and may be dependent on particle size and shape. The irregularity of TP shape may contribute to the varying retention times. Egestion of 10  $\mu$ m polystyrene microspheres in other mysid species (*N. integer*) has been observed to occur within 12 hours of ingestion (Setälä et al., 2014). Microplastic can also agglomerate with increasing salinity, leading to longer retention times in estuarine species closer to marine environments (Ogonowski et al., 2016). In a study comparing the physiological toxicity of polystyrene and carboxylate polystyrene (PS-COOH) in mysid shrimp, both plastics were observed to reduce feeding efficiency in these organisms. Future studies should carefully evaluate changes in density and sinking rates for TP at different salinities.

We observed reduced growth in *M. beryllina* across all micro-TP and two nano-TP (6000 and 60,000 p/ml) concentrations with increasing salinity, except for individuals exposed to nano-TP at 5 PSU. Other studies have also demonstrated growth inhibition of larval fish due to ingestion and accumulation of microplastics in the gut (Athey et al., 2020; Santos et al., 2020). *A. bahia* appeared to be less sensitive to growth restriction by TP and exhibited a reduction in growth only in micro concentrations at 20 PSU and 25 PSU. Nano-TP did

not elicit a significant response in growth reduction in *A. bahia*. Inhibited growth in another species, *N. japonica*, has been observed as a result of chronic polystyrene exposure (Lee et al., 2021). Several studies investigated the effect that microplastics have on the growth of small aquatic organisms. Though some studies found that microplastic, particularly polyethylene, exposure did not affect growth (Malinich et al., 2018; Mazurais et al., 2015), others noted detrimental effects of microplastics on growth (Athey et al., 2020; Lee et al., 2021). These contrasting reports may be attributed to the wide variety of microplastic compositions, shapes, and sizes, as well as the lengths of exposure. While no studies could be found in the current literature on the effects of TP on mysid or silverside growth, the findings of this study are in line with toxicity assessments for other microplastics. For example, exposure of mysids (*N. japonica*) to polystyrene (PS) and PS-COOH resulted in growth inhibition in a dose-dependent manner in which increasing concentrations resulted in decreasing growth (Wang et al., 2020). Additionally, mysids (*N. awatschensis*) showed impaired growth when exposed to melamine resin microparticles over four weeks (Lee et al., 2021). For amphipod (*H. Azteca*), chronic exposure to polyethylene microplastic particles and acute exposure to polypropylene microplastic fibers significantly decreased growth (Au et al., 2015). Similarly, growth inhibition in larval fish has been documented as an effect of exposure to micro polyvinyl chloride (Xia et al., 2020), low density polyethylene (Athey et al., 2020) and microplastic mixtures (Naidoo and Glassom, 2019; Pannetier et al., 2020). Furthermore, a meta-analysis of the literature (Foley et al., 2018) found that overall, exposure of zooplankton to microplastics decreases growth, and food dilution is thought to be one of the major mechanisms of MP toxicity to aquatic organisms in general (de Ruijter et al., 2020; Koelmans et al., 2020).

In aquatic environments, TPs are influenced by tidal processes, currents, and waves and may disperse throughout the estuarine system. At lower salinities closer to the river mouth, TP may remain suspended or float, which may make them more available to organisms that feed in the water column. TP and other particulates will agglomerate at higher salinities and biofouling may occur, increasing the potential for higher density particles to settle out into benthic environments. As mysids are indiscriminate feeding epibenthic organisms, this settling out may increase the likelihood that mysid shrimp occurring at higher salinities will encounter and ingest TP. Additionally, mysid shrimp are confirmed to ingest MP through their prey (Setälä et al., 2014). TP is likely to follow the same fate of planktonic trophic transfer. Inland silversides typically feed in the water column on copepods, mysids, and other zooplankton, although bottom feeding has been observed (Weinstein, 1986). In this respect, inland silversides may ingest TP in both the demersal and benthic environments. Future studies should further investigate the estuarine processes that affect TP circulation and transport and how this will impact aquatic species.

#### 4. Conclusion

Following exposure, *M. beryllina* and *A. bahia* had significantly altered swimming behaviors, such as increased freezing, changes in positioning, and total distance moved, which could lead to an increased risk of predation and foraging challenges in the wild. Growth for both *A. bahia* and *M. beryllina* was reduced in a concentration-dependent manner when exposed to micro-TP, whereas *M. beryllina* also demonstrated reduced growth

when exposed to nano-TP (except lowest concentration). The specific effects of particles on growth in our study are notable, in comparison to the insignificant effect of leachate on growth. TP internalization was dependent on the exposure concentration and to some extent salinity in both taxa. Recently the role of behavioral ecotoxicology in environmental conservation has been discussed by various scholars (Ford et al., 2021). This includes lab-based research that can help to study more about the individual, population, and ecosystem processes. Our research demonstrated the occurrence of significant behavioral changes in response to the lowest concentration (60 particles/ml), as well as to higher potential future concentrations and leachates under various salinities found in the estuarine environment. These behaviors represent ecologically important stimulus responses in field conditions, including activity (movement, velocity, freezing), boldness (in zone duration and frequency), and exploration (meander, turn angle, distance moved). Behavioral responses connect directly to population fitness and ecosystem-level impacts, therefore carrying high relevance to be considered by policymakers. Additionally, growth and TP ingestion data represent the significant impacts of micro and nano-TP on both of these model species that may have population-level implications. Specifically, data collected in the presence of estuarine conditions over different salinity gradients, that can aid in the assessment of risk over wider environmental ranges. Although automobiles are here to stay, limiting TP from entering the environment is paramount if we wish to preserve sensitive aquatic ecosystems and fisheries. Possible actions to take in order to achieve this goal may include providing incentives for citizen awareness of and participation in waste reduction (Eriksen et al., 2014, p. 201; Rochman et al., 2021) redesigning tire constituents with biopolymers and materials for circularity (Karan et al., 2019) extending tire producer responsibility for the end of life products, (Leal Filho et al., 2019) improving wastewater treatment technology, (Edo et al., 2020; Katyal et al., 2020) and passing legislation to ban certain synthetic materials, as well as increasing use of public transportation rather than single vehicle use (Deng et al., 2020).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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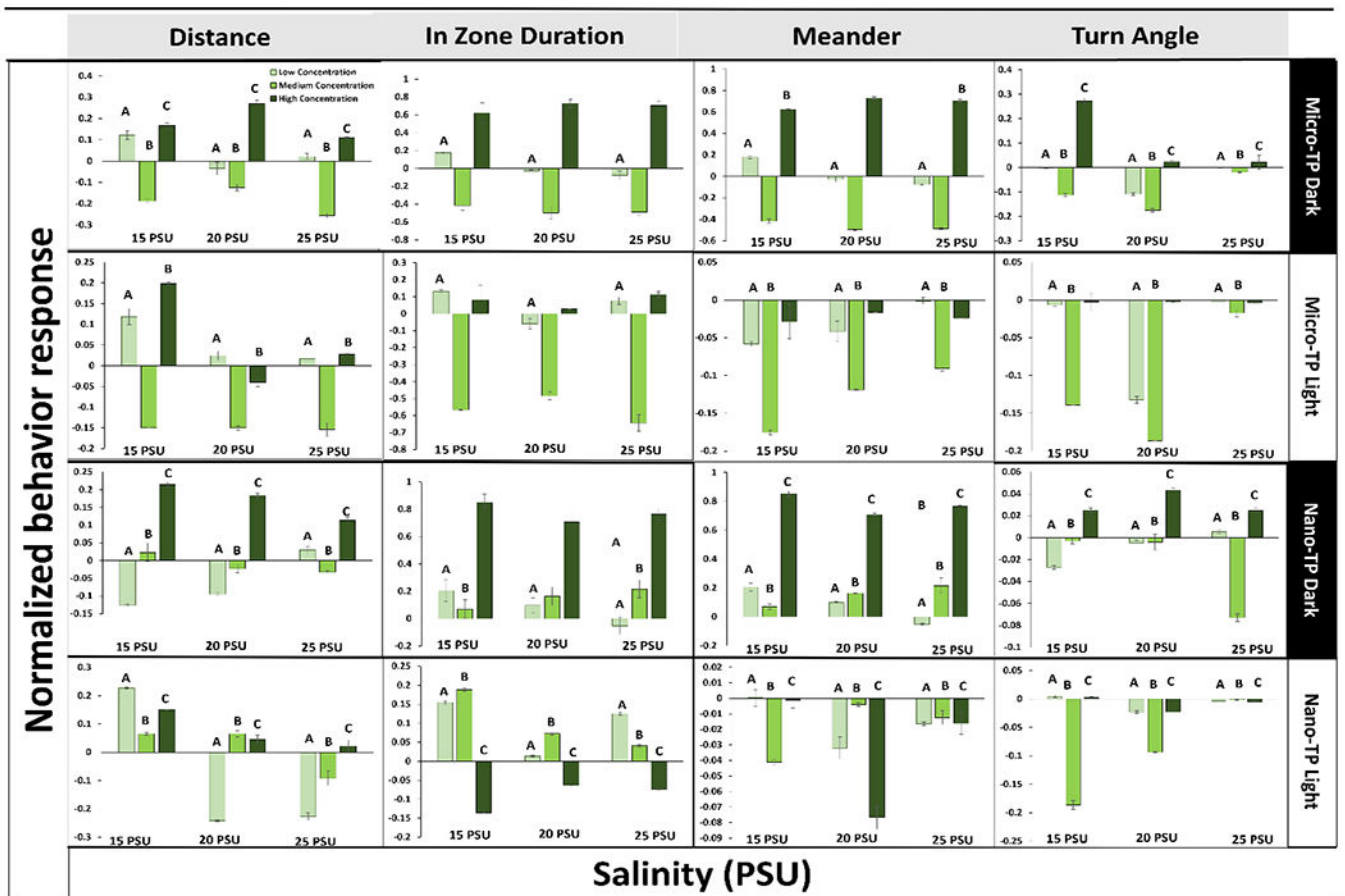
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### Highlights

- Larval mysid shrimp and silversides had significantly altered swimming behaviors.
- Growth was reduced in both species in a concentration dependent manner in  $\mu$ TP.
- TP internalization was dependent on the exposure salinity in both taxa
- *A. bahia* & *M. beryllina* ingested significantly more particles at 15 PSU in  $\mu$ TP



**Fig. 1.**

Mysid shrimp (*A. bahia*) behavioral responses with selective significant variables, represented after 7 days exposure to micro and nano-TP in combined average dark and light cycles 3 concentrations (60, 6000, 60000 particles/ml) (Lighter to darker color represents lowest to highest concentration) of across a salinity gradient 15PSU – 25PSU. Y-axis represents data normalized to 0-1 scale. Similar alphabets represent statistically significant difference in at least one salinity (\*  $p < 0.05$  ANOVA test followed by Dunnet's test, comparing all concentrations to their respective salinity NOM control within each cycle per salinity (Control = 0)). Lighter to darker color represents lowest, medium and highest concentration.

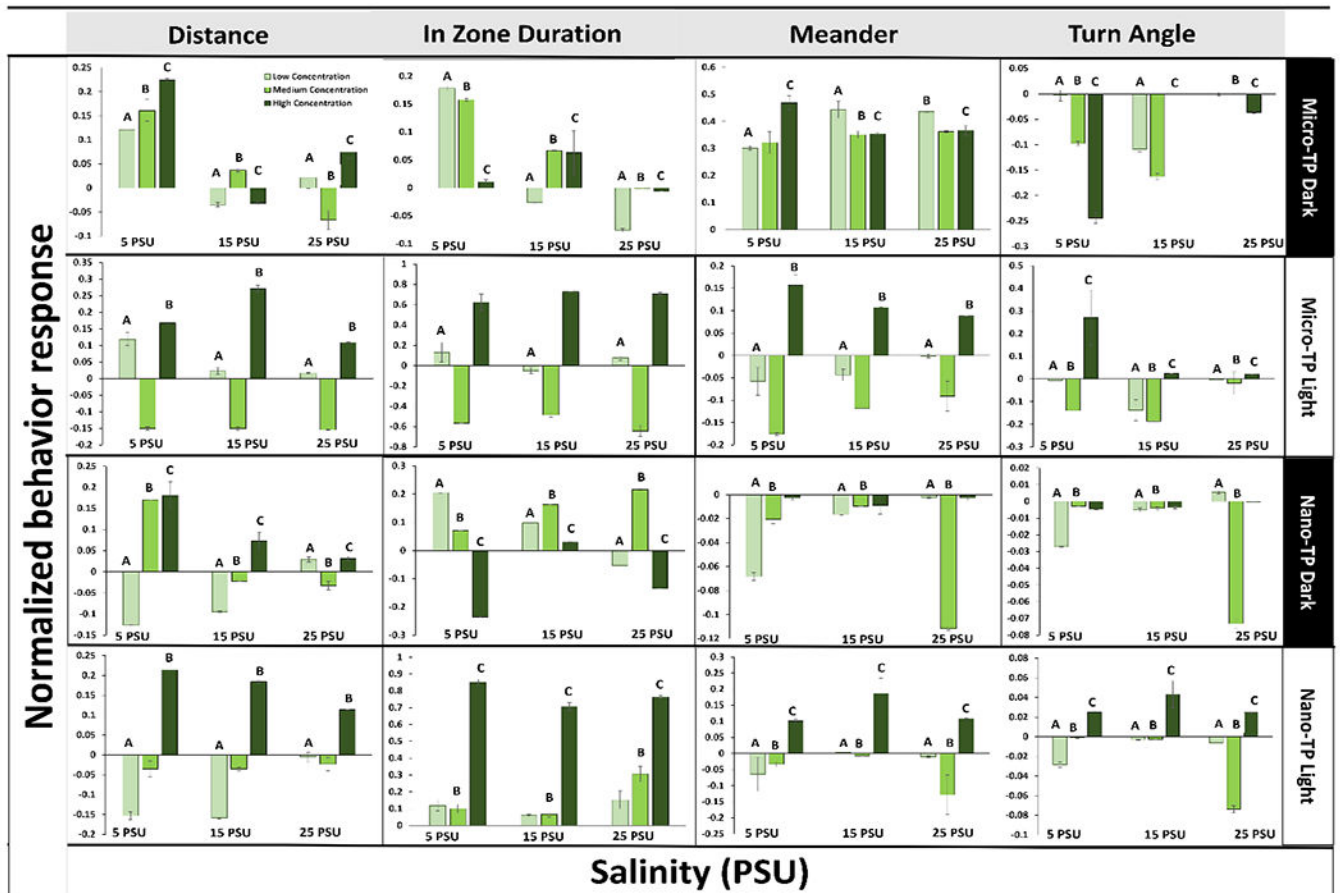
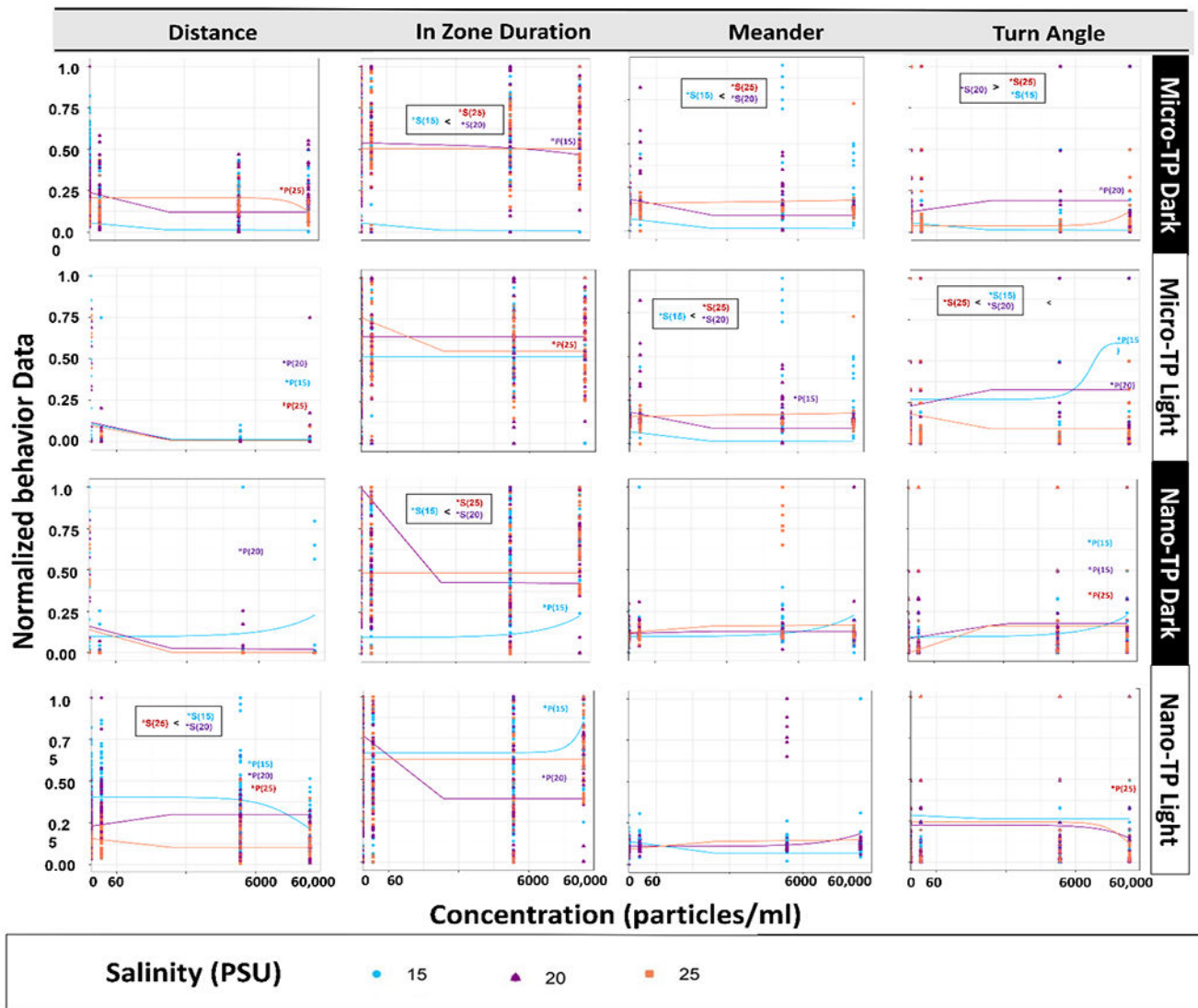


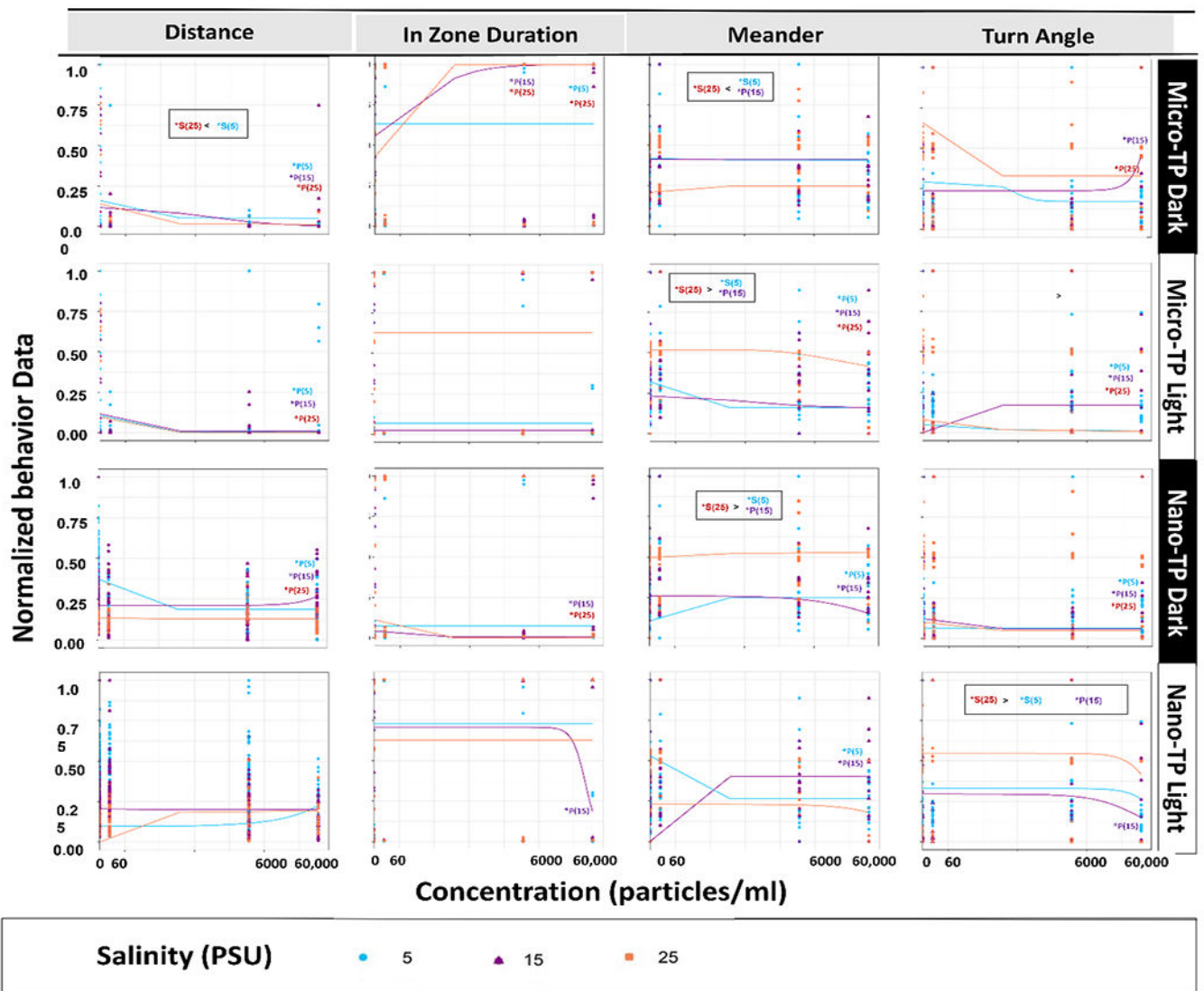
Fig. 2.

Silverside (*M. beryllina*) larvae behavioral responses with selective significant variables, represented after 4 days exposure to micro and nano-TP in combined average dark and light cycles 3 concentrations (60, 6000, 60000 particles/ml) (Lighter to darker color represents lowest to highest concentration) of across a salinity gradient 5PSU – 25PSU. Y-Axis data normalized to 0-1 scale. Similar alphabets represent statistically significant difference in at least one salinity (\*  $p < 0.05$  ANOVA test followed by Dunnet's test, comparing all concentrations to their respective salinity NOM control within each cycle per salinity (Control = 0)). Lighter to darker color represents lowest, medium and highest concentration.

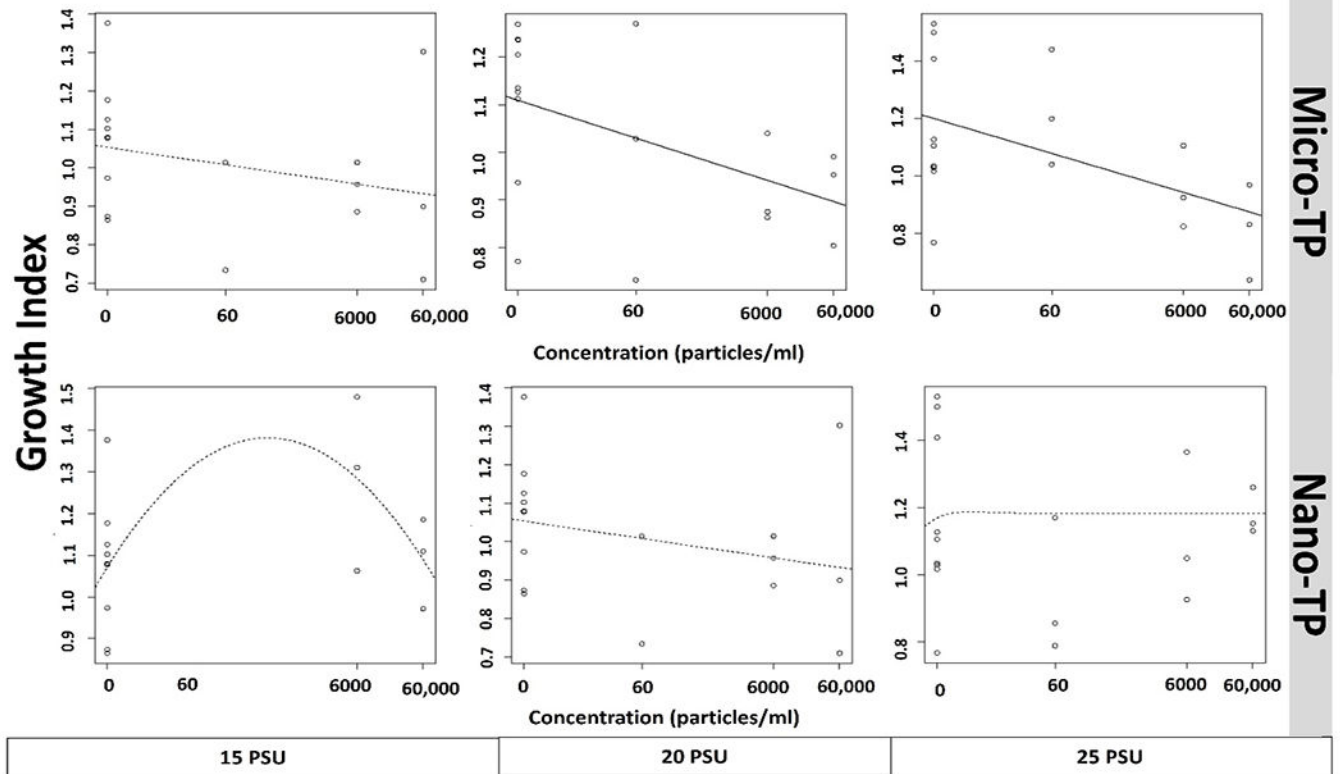


**Fig. 3.** Mysid shrimp (*A. bahia*) behavioral concentration response curves after 7 days exposure to micro and nano-TP in combined average dark and light cycles across a salinity gradient 15PSU – 25PSU. “P” represents particle count and “S” salinity. Data normalized to 0-1 scale. \*  $p < 0.05$  ANOVA test followed by Dunnet’s test, comparing all concentrations to their respective salinity NOM control within each cycle per salinity.



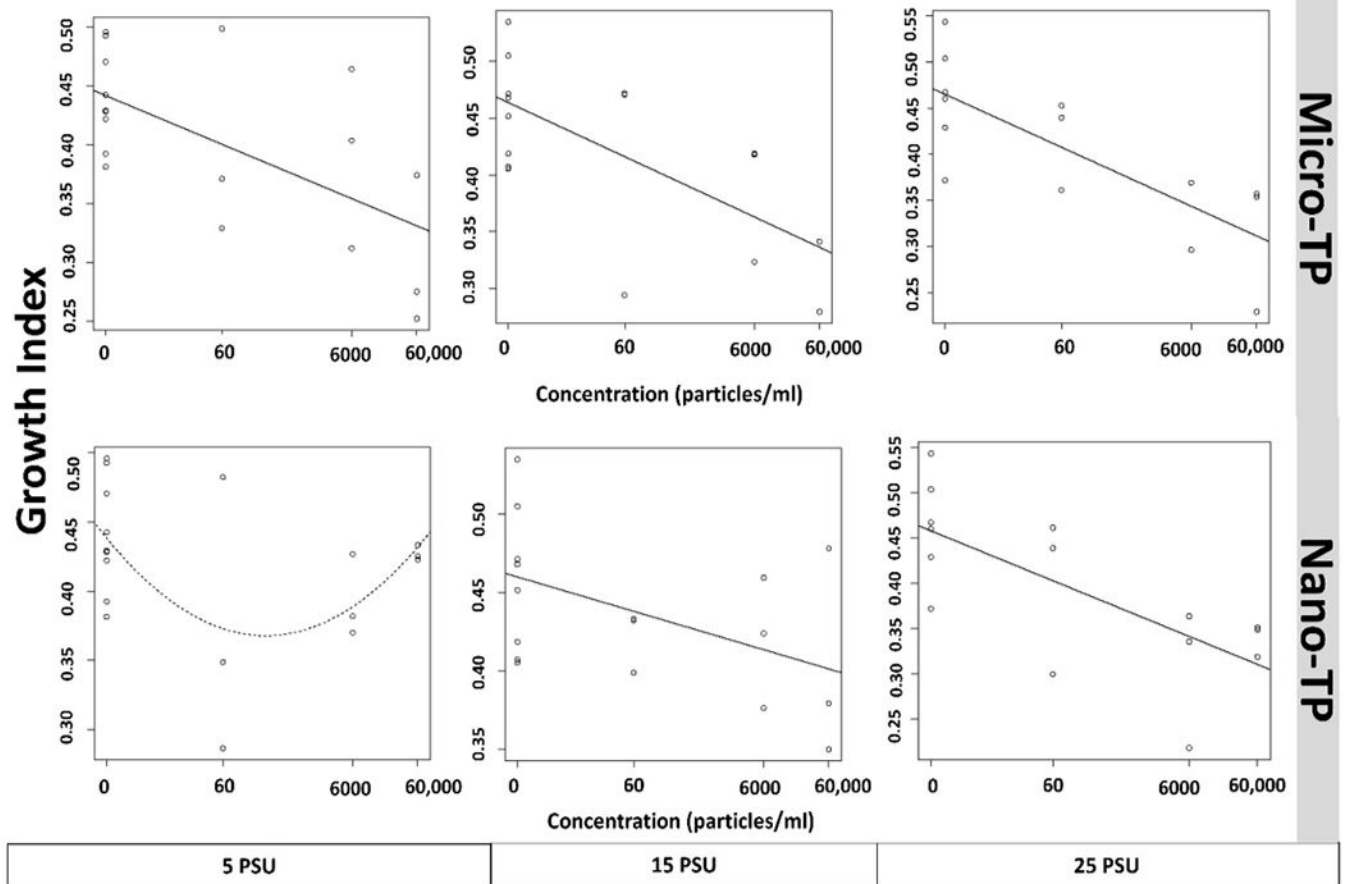


**Fig. 4.** Silverside (*M. beryllina*) larvae behavioral concentration response curves after 7 days exposure to micro and nano-TP in combined average dark and light cycles across a salinity gradient 5PSU – 25PSU. “P” represents particle count and “S” salinity. Data normalized to 0-1 scale. \*  $p < 0.05$  ANOVA test followed by Dunnet’s test, comparing all concentrations to their respective salinity NOM control within each cycle per salinity.

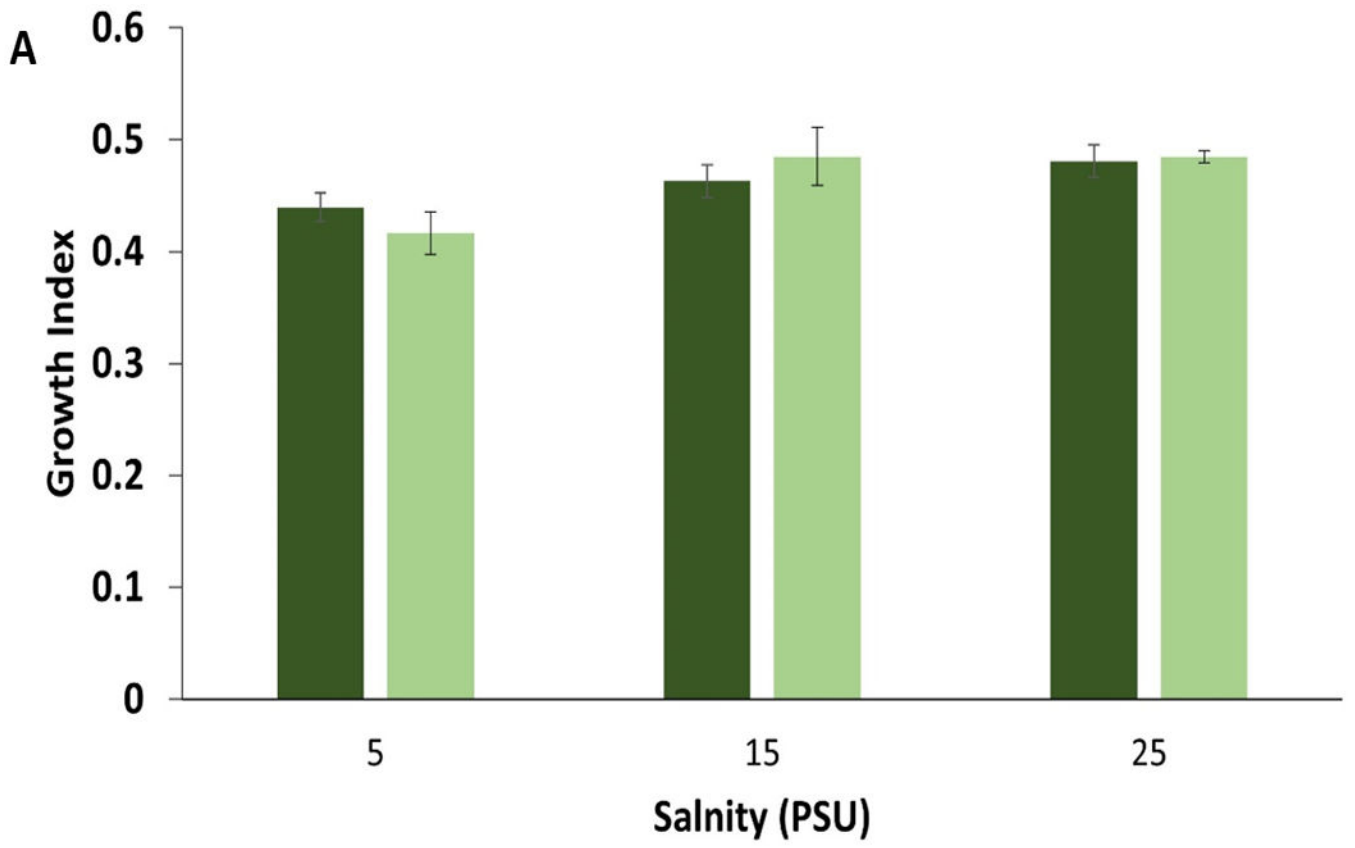


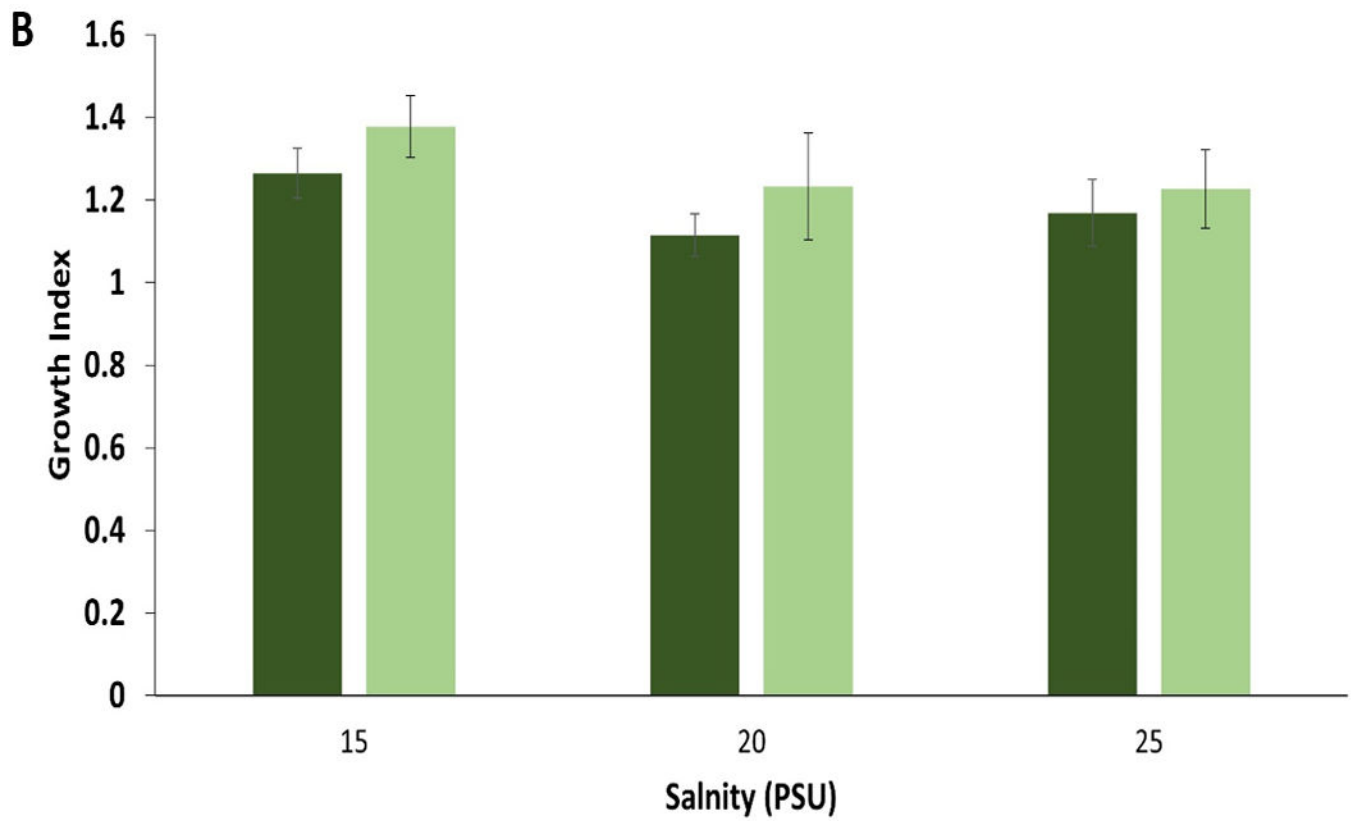
**Fig. 5.**

Concentration response growth curve of mysid shrimp (*A. bahia*) larvae exposed to micro and nano-TP across 15-25 PSU salinity gradient. Each circle represents the rescaled growth index mean of one larva (n=9). Data are presented on a  $\log_{10} X + 0.05$  axis. Curves shown as a solid line are significantly better fits than a null intercept-only model ( $p < 0.05$ ), curves shown as a dashed line are the best-fit of the five-curve option (lowest p-value), but not significantly better than the null model.



**Fig. 6.** concentration response growth curve of silverside (*M. beryllina*) yolk-sac larvae exposed to micro and nano-TP across 5-25 PSU salinity gradient. Each circle represents the rescaled growth index mean of one larva (n=6). Data are presented on a  $\log_{10} X + 0.05$  axis. Curves shown as a solid line are significantly better fits than a null intercept-only model ( $p < 0.05$ ), curves shown as a dashed line are the best-fit of the five-curve option (lowest p-value), but not significantly better than the null model.





**Fig. 7.** Control (dark color) compared to leachate (Light color) growth bar plot for A) mysid shrimp (*A. bahia*) and B) silverside (*M. beryllina*) yolk sac larvae from micro-TP concentration at 5-25 PSU salinity gradient range.

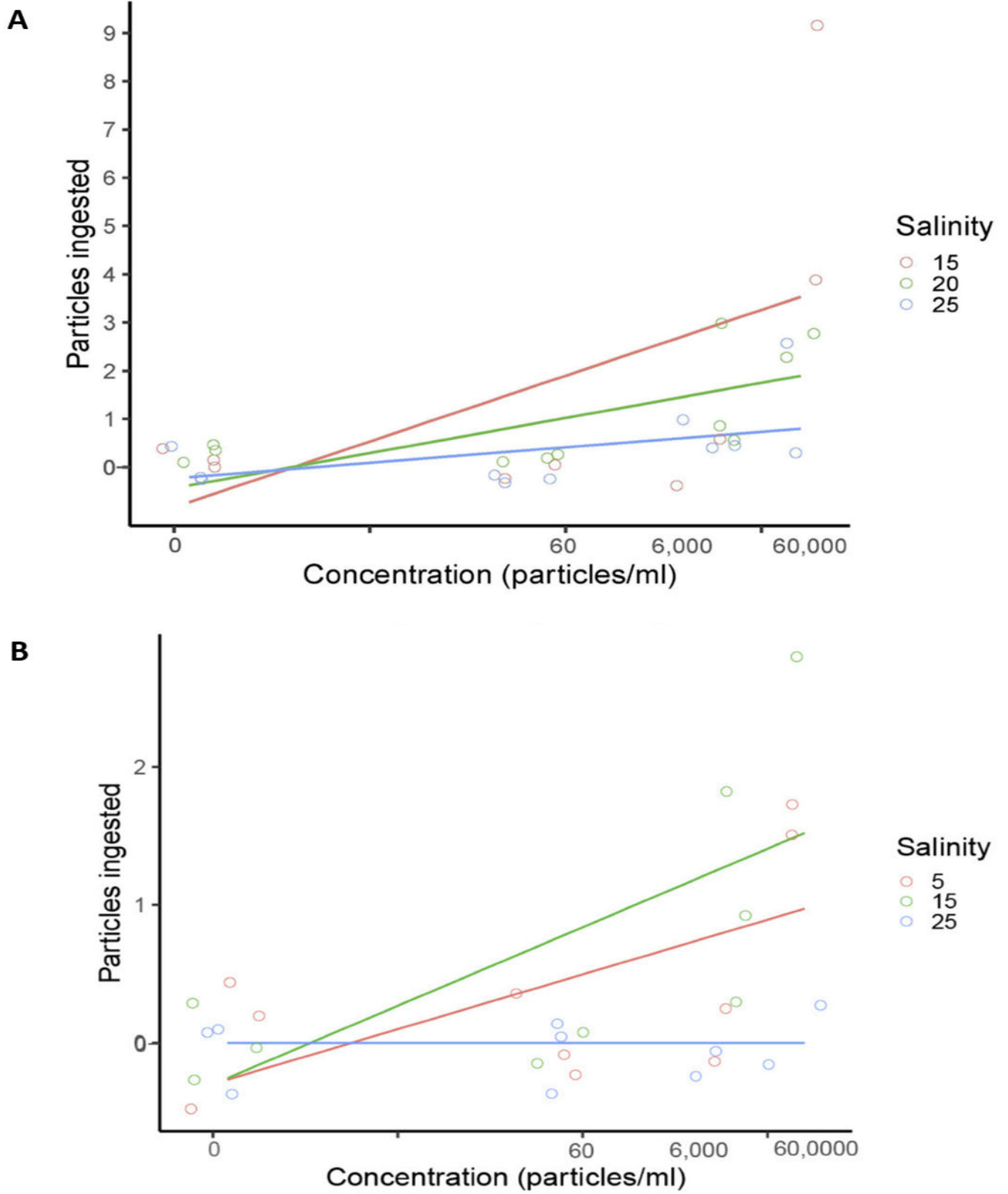


Fig. 8.

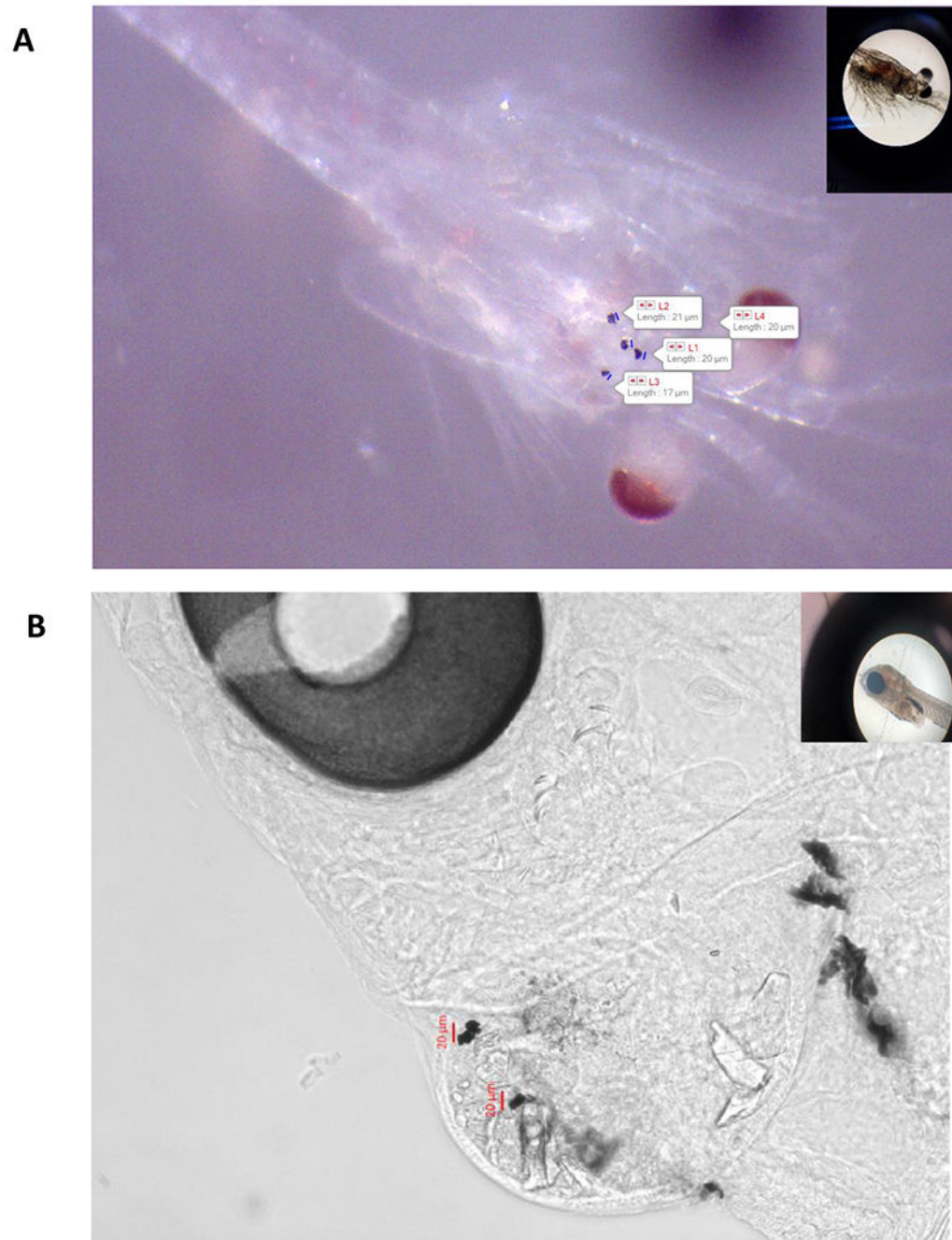
Generalized linear model of TP ingested by A) mysid shrimp (*A. bahia*) and B) silverside (*M. beryllina*) yolk sac larvae from micro-TP concentration at 5-25 PSU salinity gradient range.

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**Fig. 9.** Images of A) mysid shrimp (*A. bahia*) at 15 PSU salinity with highest micro-TP concentrations at 35x and B) larval silverside (*M. beryllina*) yolk sac at 10x exposed to highest micro-TP concentration at 5 PSU salinity. Inset images in each panel are showing control organisms.



**Table 1**

Behavioral variables from Noldus ethovision software used in this study to analyze Mysid shrimp (*A. bahia*) larvae and silverside (*M. beryllina*) larvae behavioral response

Variable	Unit	Description
Distance (Total)	cm	Total distance moved inside the well throughout the video recording time.
Freezing	S	The mean of the total time fish were moving for less than 2 seconds.
Movement	S	Duration for which the selected body point (head and tail region) was changing location with respect to the body center.
In Zone duration	S	The total time spent in the zone defined as the central portion of the beaker
In Zone Frequency		The number of times fish spent time in the zone
Meander	Deg/cm	Turning in animals moving at different speed.
Turn Angle	degree	Difference in heading between two samples.