Physiological responses of the Eastern Oyster, *Crassostrea virginica* when exposed to environmentally relevant microplastic concentrations

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Introduction

Global plastic production has increased exponentially since the 1950's and in 2017 plastic production levels were projected to have reached 348 million tons (Zhang et al., 2019). Approximately 8 million tons of plastic is estimated to enter our oceans annually with 5.25 trillion plastic particles currently circulating within our marine waters (Smith et al. 2018). Plastics can enter our waters from both terrestrial and aquatic sources. Terrestrial sources contribute roughly 80% of the plastic found in our oceans and typically come from domestic or industrial wastewater systems, stormwater runoff, or litter dropped in coastal areas or near rivers and streams (Cole et al., 2011). Aquatic sources include recreational and commercial vessels (both accidental littering and dumping), and lost or discarded fishing gear (Hantoro et al., 2019). Once within the marine environment these plastics may be broken down into smaller plastics via processes such as UV radiation, mechanical transformation (physical abrasions like wave action), and biological degradation (microorganisms). Alternatively, small plastic particles may be released directly into the environment from their source (i.e. exfoliating beauty products, toothpaste, fibers shed from clothing, etc.) (Cole et al., 2011). Plastic particles that are less than 5 mm in size can be classified as microplastics. The distribution of microplastics within the marine environment can be influenced by a variety of factors such as ocean currents, wind mixing, and the physical characteristics of the plastics themselves (Lusher, 2015). Plastics with relatively higher densities such as Polyester (1.38 g/cm^3) will readily sink within the water column and further increase the bioavailability of these microplastics to benthic organisms such as oysters (Hamm & Lenz, 2021). The impacts of microplastics on filter feeding organisms, such as the Eastern Oyster (*Crassostrea virginica),* are of particular concern given their ecological and commercial importance, as well as their remarkable ability to filter large quantities of water (up to 50 gallons of water per day). The potential risks of microplastics to oysters may include: impacts on filtering ability, feeding, reproduction, cell integrity, as well as indirect effects such as impacts on bivalve food sources, or the introduction of harmful chemicals or pathogens (Zhang et al., 2019).

Though microplastic exposure studies are no longer a rarity in the scientific community, there is a substantial need to improve the quality and environmental relevance of such studies. The majority of studies that have been conducted thus far have exposed species to high loads of microplastics (0.25-2,500,000 µg/L, 10-2.0 x 10^6 plastics/L) in order to determine the effects the plastics may have on an organism (Zhang et al., 2019). Though this may be necessary for proof-of-concept and to generate data to assess the *potential* risk of this new class of contaminants, such concentrations have been found to be two to seven orders-of-magnitude higher than what would typically be found within the natural environment (Lenz et al., 2016). There have been several attempts at utilizing environmentally relevant microplastic concentrations in exposure studies thus far with conflicting results (Bour et al., 2018; Revel et al., 2020). Discrepancies in previous studies demonstrate the need to investigate species specific responses as they are likely inconsistent even within environmentally relevant scenarios. The proposed study intends to contribute to the limited knowledge regarding how environmentally relevant exposure to

microplastics and microplastics laced with pollutants impacts the physiology of the commercially important species *C. virginica.*

Methods

Experimental Setup

C. virginica ranging from 45-54 mm in size (approximately 2 years old) were obtained from a local site with low loading of microplastics. The oysters were then placed in acclimation tanks with recirculated (with a 1 μm cartridge filter to remove any water borne plastic contamination) seawater at ambient temperature and salinity conditions for 2 weeks. During acclimation, oysters were fed a mixed algal diet (Shellfish Diet 1800, Instant Microalgae, Reed Mariculture) of approximately 100,000,000 cells per oyster daily. Following acclimation, oysters were then moved into the experimental tanks and exposed to two concentrations of microplastics (1 fiber/ml and 10 fibers/ml) both with and without the presence of pollutants (Polychlorinated biphenyl i.e. PCBs) for 3 months (Table 1). The experimental exposure consisted of 4 independent treatments and a control group with 8 replicate tanks each. Experimental conditions will be referred to by their respective treatment name rather than fiber concentration throughout this report to enhance readability. Throughout the experiment, environment variables such as water temperature, salinity, pH, ammonia, nitrite, nitrate, and mortality were measured on a daily basis.

Table 1. Summary of experimental treatments and their corresponding polyester fiber concentrations.

Treatment	Fibers/mL seawater
Baseline	0
Control	0
[Fiber 1]	1
[Fiber 1] + PCB	1
[Fiber 2]	10
[Fiber 2] + PCB	10

Microplastics

This experiment utilized small polyester fibers (avg. length 663 μm, avg. width 18 μm) for our microplastic exposures as fibers are one of the most common forms of microplastic pollution found within the marine environment (De Sá et al., 2018). Fibers most commonly enter our waters via laundering processes and are then transported to the marine environment via wastewater drainage systems (Hartline et al., 2016). Fibers are also shed from materials already in the marine environment via abrasion or degradation of synthetic fishing nets or ropes. Additionally, polyester fibers were selected for this study as preliminary trials determined they are able to sufficiently adsorb PCBs. It is known that plastics are capable of adsorbing organic pollutants from the surrounding seawater however, what kind of threat this poses to aquatic organisms is still very much unknown (Avio et al., 2015; Zhang et al., 2018). Polyester fibers in this experiment were pre-treated with a known concentration of PCBs in order to assess the ability of the fibers to act as a potential vector for this pollutant.

Sample Collection

Whole body oyster tissue samples were collected from *Crassostrea virginica* before the experiment had begun (referred to as the baseline measurements) and once again after the 3 month experimental exposure. A total of 8 oysters were collected and measured (shell length) from each treatment group for use in physiological testing. Tissue samples were rapidly frozen with liquid nitrogen and stored at -80°C. Once ready for processing, tissue samples were ground and homogenized.

Physiological Measurements

In order to assess the physiological health of *C. virginica,* cellular energy allocation (CEA) was determined in order to quantify the scope for growth (i.e. balance between energy acquired and energy expended) among oysters exposed to the various experimental treatments (De Coen & Janssen 1997; Erk et al., 2011). The cellular energy protocol was based upon methods described within De Coen & Janssen (1997) and consisted of 4 types of assays to measure the energy fractions within *C. virginica* cells. Carbohydrates (Trichloroacetic acid and glycogen assay), proteins (Bicinchoninic acid protein kit), and lipid assays (sulpho-phospho-vanillin assay) represent the energetic resources available to the oysters and measurements of mitochondrial activities (i.e. measuring the electron transport system (ETS) enzyme activity via a maximum potential activity assay) represent the energetic demands (i.e. energy consumed (Ec)) of the oysters. At the end of each assay, tissues samples were measured spectrophotometrically (Thermo Scientific, Multiskan FC microplate spectrometer) and readings were then converted into their energy equivalent using the energy of combustion values from Gnaiger (1983); glycogen 17500 mJ/mg, protein 24000 mJ/mg, lipid 39500 mJ/mg. These values representing the energetic resources were then combined to calculate the energy available (Ea) to the organisms. The overall cellular energy allocation to the oysters was determined by dividing Ea by Ec.

Statistical Analysis

All data was analyzed using R Studio (version 4.0.5) statistical software. Growth, carbohydrate, lipid, protein, Ea, Ec, and CEA values were all tested for normality by generating Q-Q plots for each respective treatment group. For the sake of this assignment, normality was assumed for all data. A one-way ANOVA was used to compare the means of each experimental treatment group in order to determine if there were any significant differences amongst all of the groups. If ANOVA results indicated the presence of a significant difference among experimental groups, a post hoc analysis was then conducted. The Tukey's honestly significant difference test (Tukey HSD) was chosen as the post hoc analysis to identify which experimental treatment groups (if any) were significantly different from the baseline measurements.

Results

Growth

All treatment groups appeared to have experienced growth in shell length throughout the duration of the experiment. Final shell lengths averages were similar for [Fiber 1], [Fiber 1] + PCB, [Fiber 2], & the control group whereas the shell length average in [Fiber 2] + PCB was smaller overall (Table 2). Results from ANOVA testing indicate a significant difference in growth among the experimental treatments (ANOVA, $df = 5$, $p = 4.41 \times 10^{-15}$). Results from Tukey HSD test indicate that differences between all experimental treatments and

the baseline measurements are significant ($p < 0.05$) with the exception of the [Fiber 2] + PCB group ($p = 0.139$) (Figure 1).

Table 2. Summary of mean shell lengths for each experimental treatment group. Baseline measurements were taken before the experiment began. Remaining treatment measurements we taken at the end of the exposure experiment (3 months after baseline measurements).

Treatment	Mean Shell Length (mm)
[Fiber 1]	54.7500
[Fiber 1] + PCB	54.6375
[Fiber 2]	54.8375
[Fiber 2] + PCB	48.7625
Baseline	46.7125
Control	54.6500

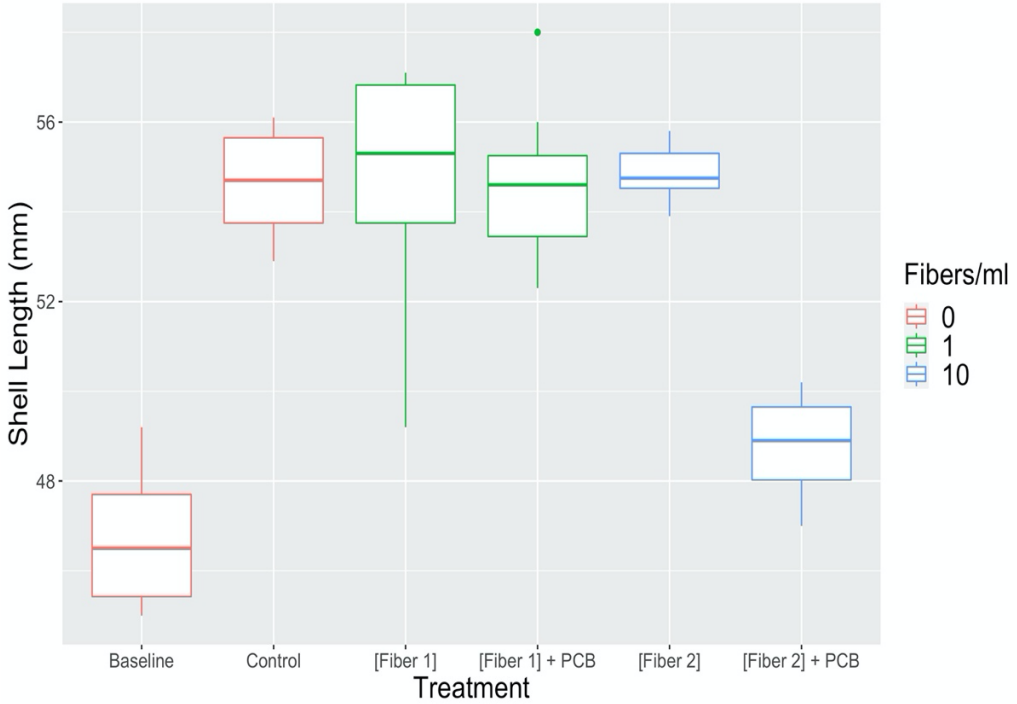


Figure 1. Boxplot containing shell length of oysters (n = 8 per treatment). Baseline measurements represent shell length at the beginning of the experiment. All other lengths were measured 3 months after the baseline measurements, at the end of the experiment. Colored outlines indicate the actual fiber concentration of each treatment (red = 0 fibers/ml, green = 1 fiber/ml, blue = 10 fibers/ml).

Mortality

When oysters were exposed to PCBs the number of mortalities increased regardless of the fiber concentration. The [Fiber 2] + PCB treatment group had the highest number of mortalities overall (n = 7), followed by [Fiber 1] + PCB (n = 6) and [Fiber 1] (n = 1). The [Fiber 2] and control groups had no mortalities over the duration of the experiment (Figure 2).

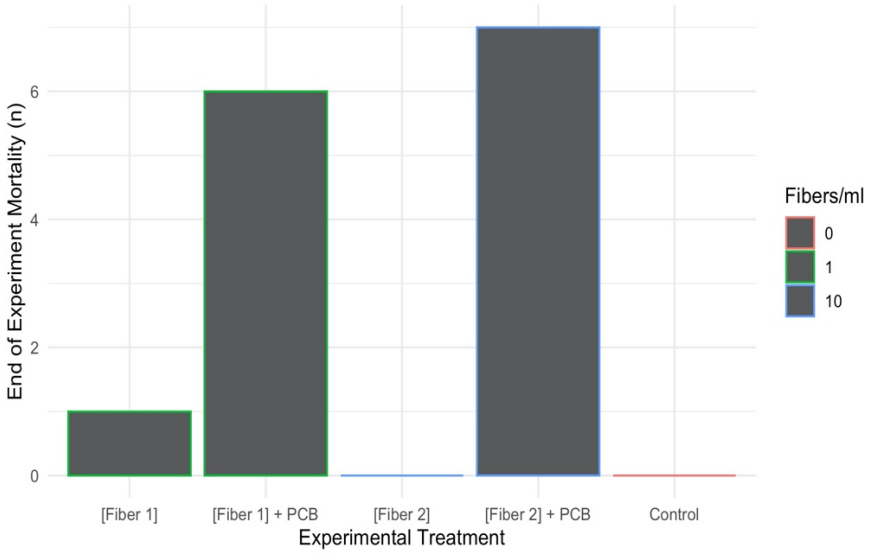


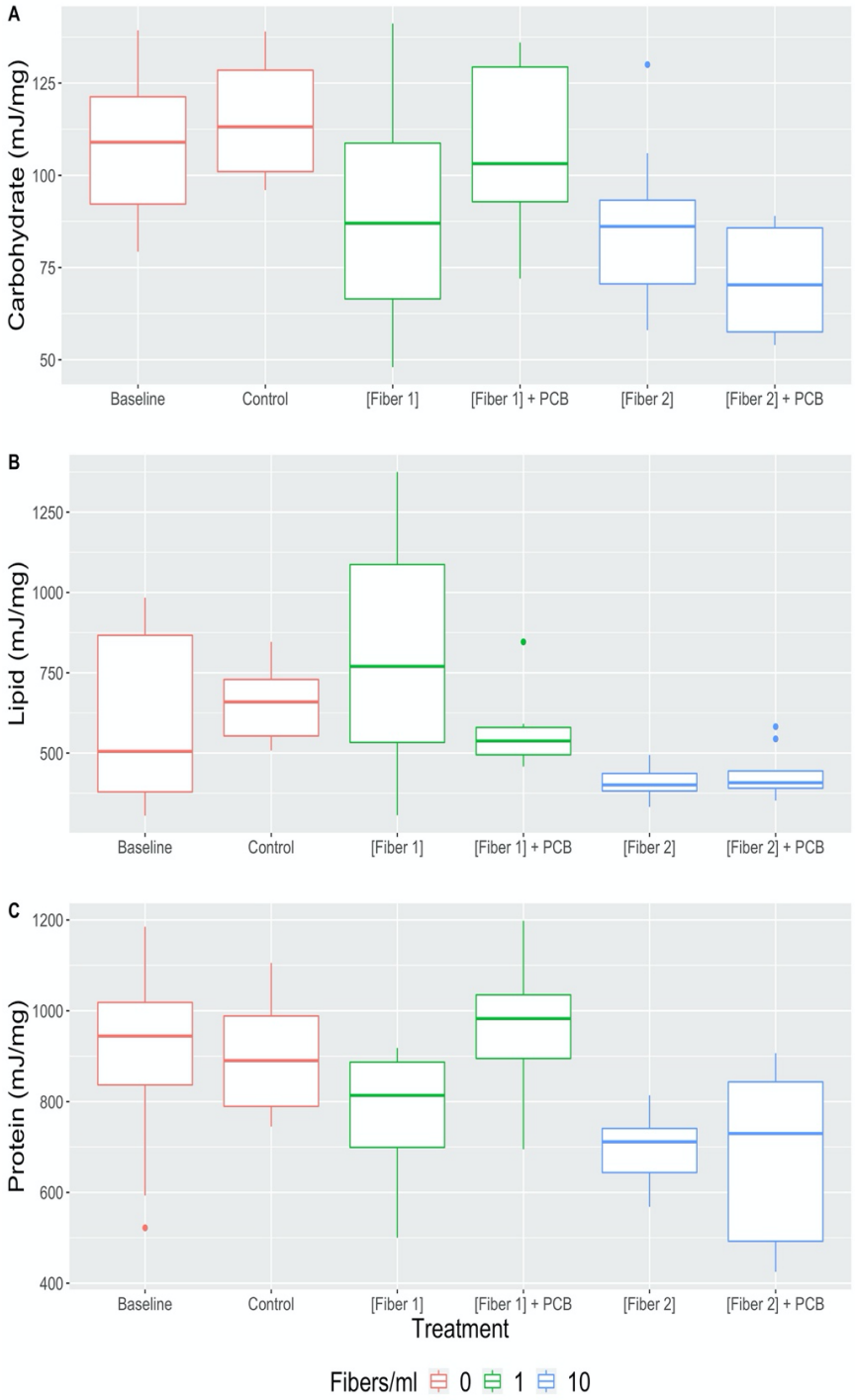
Figure 2. End of experiment mortality for each of the 5 experimental treatments. Colored outlines indicate the actual fiber concentration of each treatment (red = 0 fibers/ml, green = 1 fiber/ml, blue = 10 fibers/ml). Experimental treatments that contained fibers laced with PCBs experienced higher moralities than those exposed to fibers only. There were no moralities within the control group over the duration of the experiment.

Cellular carbohydrate, lipid, & protein content

Total energy from cellular carbohydrate content appeared lower within the [Fiber 2] concentrations (Figure 3). Results from ANOVA indicate a significant difference among the various treatment groups (ANOVA, df = 5, p = 0.0034). A Tukey HSD analysis revealed that there were no significant differences among any of the treatment groups when compared to baseline cellular carbohydrate content with the exception of the [Fiber 2] + PCB treatment (p = 0.022) (Figure 3).

Total energy from cellular lipid content also appeared to be slightly lower within the [Fiber 2] concentrations. Results from ANOVA indicated a significant difference in lipid content amongst the various treatment groups (ANOVA, df = 5, p = 0.0038). Subsequent Tukey analysis revealed that there were no significant differences among any of the treatment groups when compared to baseline cellular lipid content (Figure 3). Significant differences detected in ANOVA test likely from inter-treatment comparisons rather than treatment-baseline comparisons.

Total energy from cellular protein content also appeared to lowest overall within the [Fiber 2] concentrations. Results from ANOVA indicated a significant difference amongst the various treatment groups (ANOVA, df = 5, p = 0.0053). Subsequent Tukey analysis revealed no significant differences among any of the treatment groups when compared to baseline cellular protein content (Figure 3). Significant differences detected in ANOVA test likely from inter-treatment comparisons rather than treatment-baseline comparisons.



Carbohydrate Content

Contrast	p value
Baseline-[Fiber 1]	0.6030048
Baseline-[Fiber 1] + PCB	0.9999873
Baseline-[Fiber 2]	0.4023310
Baseline-[Fiber 2] + PCB	0.0224792
Control-Baseline	0.9943546

Lipid Content

Contrast	p value
Baseline-[Fiber 1]	0.3219487
Baseline-[Fiber 1] + PCB	0.9995116
Baseline-[Fiber 2]	0.4709599
Baseline-[Fiber 2] + PCB	0.6106709
Control-Baseline	0.9903939

Protein Content

Contrast	p value
Baseline-[Fiber 1]	0.6349622
Baseline-[Fiber 1] + PCB	0.9860240
Baseline-[Fiber 2]	0.1590206
Baseline-[Fiber 2] + PCB	0.1155744
Control-Baseline	1.0000000

Figure 3. Total energy (mJ/mg) derived from cellular nutrient content extracted from whole body *C. virginica* tissue samples and their respective results from Tukey significance tests. Baseline measurements were conducted before the experiment began. The remaining treatments were sampled at the end of the exposure experiment (3 months later). Colored outlines indicate the actual fiber concentration of each treatment (red = 0 fibers/ml, green = 1 fiber/ml, blue = 10 fibers/ml). **A)** Total energy from cellular carbohydrate content (mJ/mg). Tukey significance testing indicated that differences in carbohydrate content between the [Fiber 2] + PCB treatment and baseline measurements are statistically significant ($p = 0.022$). **B)** Total energy from cellular lipid content (mJ/mg). No significant differences in lipid content among any of the experimental treatments when compared to baseline lipid measurements. **C)** Total energy from cellular protein content (mJ/mg). No significant differences among any of the experimental treatments when compared to baseline protein measurements.

Energy Available & Energy Consumed

ANOVA results indicated a significant difference in energy available (Ea) values between the experimental groups (ANOVA, df = 5, p = 0.005). Subsequent Tukey HSD analysis indicated that there were no significant differences between any of the experimental treatment groups and the baseline available energy values (Figure 4). Significant differences detected in ANOVA test likely from inter-treatment comparisons rather than treatment-baseline comparisons. ANOVA results showed no significant differences in energy consumed by the animal (Ec) among any of the experimental groups (ANOVA, df = 5, p = 0.960).

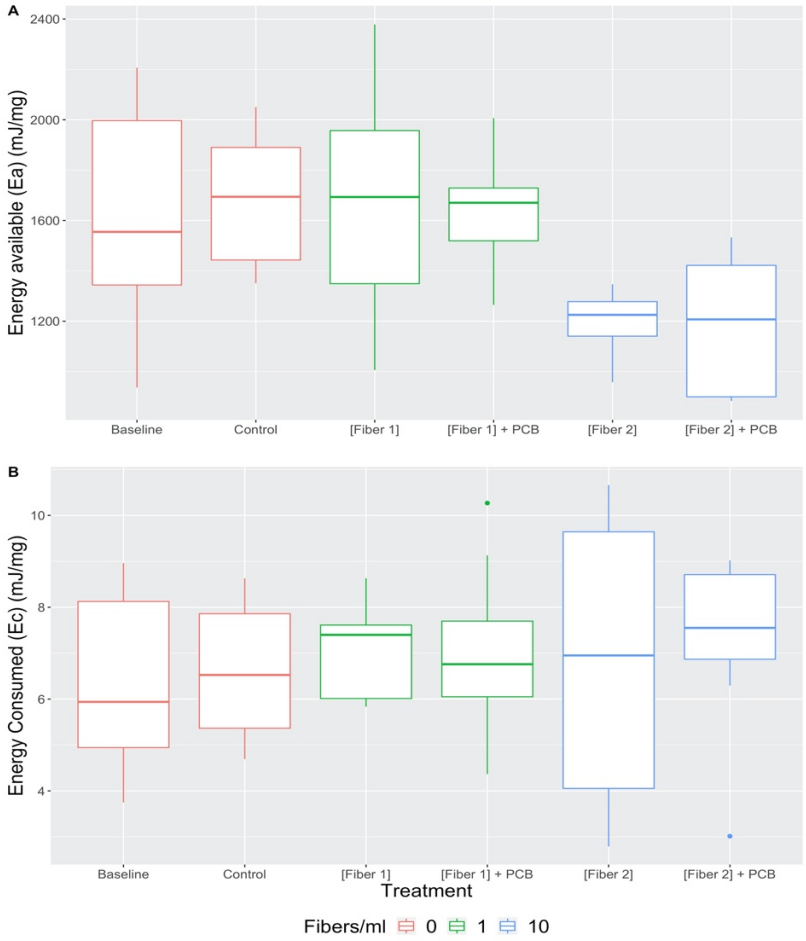


Figure 4. Plots of available cellular energy and energy consumption (reported as mJ/mg tissue weight) separated out by experimental treatment group. Baseline measurements were conducted before the experiment began. The remaining treatments were sampled at the end of the exposure experiment (approximately 3 months later). **A)** Energy available to the organism (mJ/mg) i.e. the sum of energy provided by each of the measured cellular nutrients (carbohydrates, lipids, proteins). **B)** Energy consumed by the organism (mJ/mg). Energy consumption values were derived from measuring mitochondrial activity within the oyster cells.

Cellular Energy Allocation (CEA)

ANOVA results indicate no significant differences in cellular energy allocation values between any pair of experimental treatment groups (ANOVA, df = 5, p = 0.373) (Figure 5).

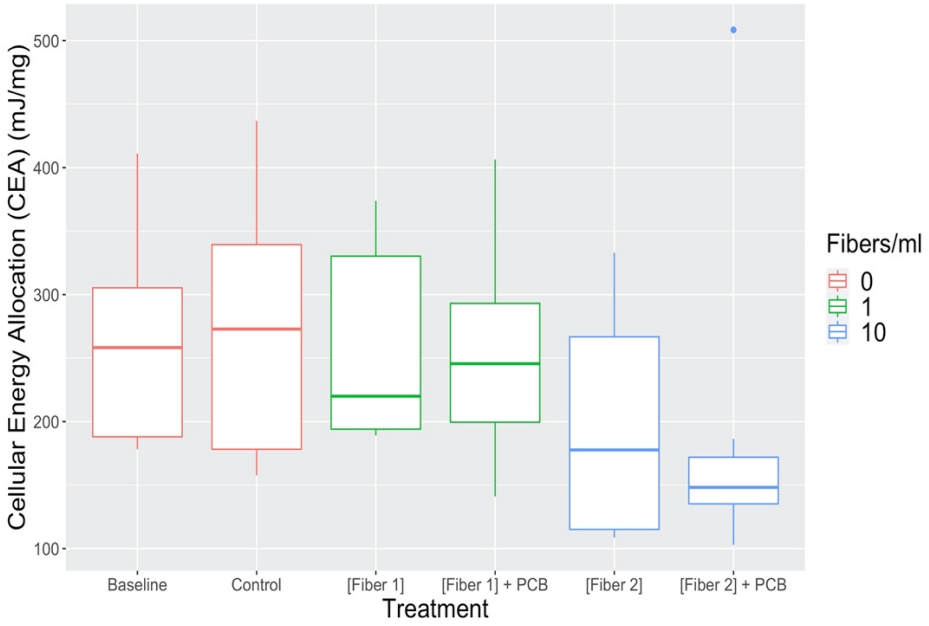


Figure 5. Cellular energy allocation values (mJ/mg) calculated by ratio of energy available (Ea): energy consumed (Ec). Baseline measurements were conducted before the experiment began. The remaining treatments were sampled at the end of the exposure experiment (approximately 3 months later).

Discussion

All experimental treatment groups experienced shell growth over the duration of the experiment when compared to baseline shell length measurements. On average, end of experiment shell length was similar across all experimental treatment groups with the exception of the [Fiber 2] + PCB treatment. Significance testing revealed that the [Fiber 2] + PCB treatment group was the only treatment group that did not have a significant difference in shell length at the end of the experiment (compared to baseline shell length values). Since the [Fiber 2] treatment did not appear to impact shell growth it is likely that the observed reduction in growth is likely due to the increased presence of PCBs within the [Fiber 2] + PCB treatment. PCBs were introduced to the experimental tanks through the polyester fibers (i.e. allowing PCBs to sorb to the fibers) so therefore an increase in fiber concentration results in an increase in PCB concentration as well. These findings support previous knowledge that organic pollutants such as PCBs have the ability to alter organismal growth (Diamanti-Kandarakis et al., 2009). These results also suggest that the relatively low (and environmentally relevant) concentration of microplastics used within our experiment does not appear to hinder the growth potential of the organisms. Such a finding is inconsistent with results of previous work (though previous studies differ in microplastic composition and concentrations which may explain discrepancies) and highlight the importance of environmentally relevant exposure conditions (Zhang et al., 2019).

Overall experimental mortality was relatively low. Mortality was substantially greater in experimental treatments that contained PCBs versus those that did not. The highest number of mortalities (n = 7) was in the [Fiber 2] + PCB treatment i.e. the treatment with the greatest amount of PCBs present. The [Fiber 1] + PCB treatment was close behind with the total mortalities adding up to n = 6. These results suggest that PCB presence is increasing mortality rates within the oysters. We are currently unable to determine whether these impacts are direct

(such as the PCBs being present in levels that are toxic to the oysters) or indirect (such as PCB presence causing physiological stress to the oysters which may make them more susceptible to other environmental stressors like poor water quality conditions or disease.

Total energy from cellular carbohydrates was lowest within the [Fiber 2] concentrations however, only the [Fiber 2] + PCB treatment had values that were significantly different from the baseline carbohydrate measurements. Overall there were no significant differences in cellular energy derived from lipids compared to baseline lipid values. Similarly, there were no significant differences in cellular energy derived from protein compared to baseline protein values. Such findings suggest that the presence of fibers and/or PCBs does not appear to impact the energy obtained by an organism through these cellular nutrients. The lowered amount of energy derived from cellular carbohydrates within the [Fiber 2] + PCB treatment may be a result of the oysters being stressed from the increased presence of PCBs. When stressed, oysters may remain shut or reduce their algae clearing (feeding) which can lead to a decrease in consumption of these essential nutrients. Inconsistencies between carbohydrates, lipids, and proteins may be due to the oysters being fed a diet of mixed algae species where each species differs in their respective cellular nutrient contents. Discrepancies may also be the result of preferential metabolism of the nutrients by the oysters.

The energy available to the oysters was determined by taking the sum of energy derived from each of the three individual cellular nutrients. Significance testing found that there were no significant differences in the available energy values present within the experimental treatment groups when compared to available energy values from the baseline measurements. Such findings indicate that the presence of the polyester fibers and/or the PCBs was not significant enough to hinder the ability of the oysters to acquire necessary cellular energy from these essential nutrients. Energy consumption values were calculated from measuring mitochondrial activity within the oyster cells. Statistical analyses revealed no significant differences in energy consumption by the oysters among any of the experimental treatment groups when compared to the baseline values. This suggests that presence of the polyester fibers and/or PCBs in these experimental concentrations does not appear to impact or suppress the metabolic rate of the oysters. Suppression of metabolic rate is typically assumed when a decrease in energy consumption is observed would suggest the oysters are exhibiting a stress response in the presence of the fiber and PCB concentrations. This lack of reduction in energy consumption suggests that the oysters were likely not significantly stressed by the experimental treatments.

The CEA values can provide information on how these energetic fractions are allocated throughout the energy budget of the oysters and allow for the quantification of the scope for growth of the organisms. Significance testing results indicate no significant differences in the overall CEA of the experimental treatment organisms compared to the baseline CEA measurements. These results indicate that overall, the polyester fibers and/or PCBs used in this experiment did not have a significant impact on the balance of energy acquired and energy consumed by the oysters. Such findings suggest that the fiber and PCB concentrations used in the current experiment will likely not influence *C. virginia* scope for growth.

The results from this experiment indicate that the concentrations of fibers and PCBs used in the present study likely do not influence the distribution of cellular energy within *C. virginia*

and therefore are unlikely to influence the organisms scope for growth. Such findings are significant as they contrast results from previous studies that have utilized much greater microplastic concentrations than oysters would likely be exposed to within the natural environment. This study highlights the need for additional exposure studies utilizing environmentally relevant microplastic concentrations in order to better understand the actual risks (if any) that microplastic pollution is posing to marine organisms such as *C. virginia* .

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