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IRVINE

Left in the dust: suspended sediment is an acute stressor for the gill, a chronic stressor for the gut of zebrafish

DISSERTATION

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Nefertiti Smith Christman

Dissertation Committee
Donovan German, Chair
Associate Teaching Professor Nancy Aguilar-Roca
Associate Professor Deborah Lutterschmidt

2025

DEDICATION

To
My parents, who have supported and encouraged me,
My sister, who has cheered for me
My husband, who has loved me through it all
My children, knowledge is something they can never take away from you

To
The unnamed and named people of color who have paved the way for me earn this accomplishment

And of course
God the All-Glorious

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Smith Christman NN., German DP. Suspended sediment as an irritant: zebrafish ate less and had reduced intestinal proteolytic activity when exposed to suspended clay. In prep, to be submitted Journal Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology

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Fong, C.R., **Smith, NN**., Catalan, E. et al. Herbivorous sea urchins (Echinometra mathaei) support resilience on overfished and sedimented tropical reefs. Sci Rep 14, 3829 (2024). https://doi.org/10.1038/s41598-024-52222-0

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ABSTRACT OF THE DISSERTATION

Left in the dust: suspended sediment is an acute stressor for the gill, a chronic stressor for the gut of zebrafish

by

Nefertiti Smith Christman

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2025

Professor Donovan P. German, Chair

Anthropogenic activities increase suspended sediment in aquatic ecosystems, posing potential chronic physiological challenges for fish. Thus, in my first chapter, I investigated the effects of chronic (28 day) exposure to suspended kaolin clay (45 mg/L) on zebrafish (*Danio rerio*), hypothesizing that ingested sediment acts as a gut perturbation leading to reduced food consumption. I assessed growth, whole-body cortisol, gill and gut histology, gill HSP60/HSP70 gene expression, and digestive enzyme activities. Sediment-exposed fish exhibited significantly shorter final body lengths and reduced relative gut lengths ($P < 0.05$ for both), supporting the hypothesis that sediment ingested with food is a gut perturbation that leads to decreased food intake. Histological analysis revealed potential increased goblet cells in the intestinal epithelium, suggesting potential heightened mucus production. Alanyl-aminopeptidase activity, vital for protein digestion, was significantly reduced ($P < 0.05$) in the fish exposed to suspended sediment. Conversely, whole-body cortisol and gill HSP expression showed no significant differences, suggesting acclimation. While preliminary due to pseudoreplication, these findings indicate that chronic suspended sediment exposure acts as a potential gastrointestinal irritant, impairing nutrient assimilation. This highlights the critical need for further robust research to understand the ecological implications of suspended sediment on fish foraging and digestion in impacted aquatic environments.

In my second chapter, I expanded on the first study. This study characterized the time-course of the physiological response of zebrafish (Danio rerio) to chronic suspended sediment exposure and their capacity for recovery. Fish were exposed to suspended kaolin clay (45 mg/L) for one, three, seven, and 28 days, with parallel recovery groups returned to sediment-free water. We assessed gill heat shock protein (*hsp60* and *hsp70*) gene expression and the activity of several digestive enzymes. The cellular stress response was transient, with significant changes in HSP expression only observed at early time points (one and seven days), indicating potential physiological acclimation. While sediment exposure had no significant effect on fish mass or length, it profoundly impacted digestive physiology. Notably, trypsin, aminopeptidase, and lipase activities were lower following sediment exposure, but appeared to rebound in the recovery fish. Thus, sediment exposure affects digestive capabilities, but can recover when suspended sediment is removed.

In my third chapter I used the same fish from chapter 2 to investigate how the gut microbiome of adult zebrafish responds to different durations of suspended sediment exposure and subsequent recovery. The gut microbiome was characterized using 16S rRNA gene sequencing. Results showed that suspended sediment caused an acute disruption to the gut microbial community. Alpha diversity (Shannon index) was significantly reduced in sediment-exposed and/or recovery groups at one and three days but not at the 28 day chronic time point. The diversity of the recovery group took seven days to become statistically indistinguishable from the control group. While overall community composition (beta diversity) did not show significant clustering, indicator species analysis revealed distinct microbial signatures for each treatment group. The control group hosted the highest number of significant indicator species, suggesting a stable baseline community that was altered by the sediment perturbation, whereas the recovery group had the most unique Amplican Sequence Variants, showing a variable microbiome. These findings indicate that suspended sediment induces a

rapid, but recoverable, loss of gut microbial diversity, and that even after diversity is restored, the underlying community composition remains altered.

INTRODUCTION

Approximately 48% of all of the world's coastal regions are developed and no coastal region is 100% intact or free from human impact (Williams et al. 2022). The maintenance and conservation of these habitats and their inhabitants remain at the forefront of importance. While our society grows, pressure on the environment increases. Due to growing technology the world becomes smaller, and we can travel between countries with ease. For example ships and boats can travel from coast to coast, but to do so, shipping channels are dredged to make way for these ships. This machinery used to dredge seabeds is a source of noise pollution known to affect aquatic organisms (Reine et al. 2014). Furthermore, when sediment is excavated there is potential for the rerelease of toxins, which is well known to have detrimental effects on aquatic organisms (Roberts 2012). In addition, sediment plumes created from dredging reduces visual acuity and behavioral avoidance (Collin and Hart 2015), which can lead to altering the structure of fish communities (Utne-Palm 2002). Another way sediment plumes become prevalent is when rain events wash sediment into local waterways. The sediment plumes are exaggerated especially on coastlines that are developed and have reduced natural flora that have roots to hold sediment in place (Sundblad and Bergström 2014).

Sediment production from anthropogenic activities have increased by about 467% between 1950 and 2010 (Syvitski et al. 2022). Smith et al. (2010) examined 4307 rivers worldwide and their changes in water and sediment fluxes, finding that 40% of the rivers had significant changes in sediment flux. For example, Li et al. (2020) observed an increase in suspended sediment in the Amazon River. Overall, anthropogenic activities, like increased sediment influx into waterways, are the biggest threat to fish health (Mallett et al. 2024). Thus it is important to study how these activities impact fish. Beyond the gill, which has been shown to be affected by suspended sediment (Hess et al. 2015; 2017), it is important to study how the digestive system, and its gut microbiome are impacted. The digestive system is vital to fish health and fitness. They need this system to ingest, digest, and distribute the nutrients from their outside environment.

Without this process they will not be able to grow, forage for food, or produce offspring. Furthermore, the gut microbiome plays a vital role in digestion, aiding in breaking down nutrients and impacting the fish immune system. Understanding whether the enteric microbial community is affected by ingestion of sediment is key to revealing the full effects of sediment exposure.

The zebrafish as a model system was chosen for many reasons. Zebrafish husbandry is well established, with clear best practices and infrastructure specifically made for this model (Lawrence 2007; Avdesh et al. 2012). The Streisinger Laboratory (University of Oregon) pioneered widespread interest in using zebrafish, capitalizing on its relatively short generation time and large egg production potential (Streisinger et al. 1981). From there, zebrafish became an ideal choice for studying mutations and annotating the genome followed (Briggs 2002). Due to the accessibility of housing zebrafish and their genome being widely studied, I am able to utilize many resources that are not available when using a non-model organism.

The first chapter of this dissertation will address the knowledge gap of long-term (28 days) physiological impacts of chronic suspended sediment exposure on fish. While it is known that sediment acts as a potential stressor, especially on the gill (Hess et al. 2015; 2017), a detailed understanding of its sublethal effects on key biological systems like digestion is lacking. I hypothesized that chronic ingestion of suspended sediment would act as a gastrointestinal irritant, leading to reduced food consumption and impaired nutrient absorption. To test this, I exposed zebrafish to a known sublethal chronic dose of suspended kaolin clay (45 mg/L) for 28 days. I measured growth, examined gill and gut histology, analyzed the activity levels of key digestive enzymes, and evaluated whole-body cortisol levels and gill heat shock protein expression. This research was designed to provide a mechanistic understanding of how suspended sediment may affect zebrafish.

Building on the findings from chapter one, the second chapter addresses the time dependent effects and physiological acclimation to sediment exposure over time. It is currently unknown how fish acclimate to chronic exposure to suspended sediment and whether the

associated physiological costs are transient or permanent. I exposed zebrafish to suspended kaolin clay (45 mg/L) for varying durations (one, three, and seven and 28 days), with parallel groups moved to sediment-free water to assess their capacity for recovery. I tracked changes in gill heat shock protein (HSP60 and HSP70) gene expression as an indicator of the cellular stress response, and measured the activity of digestive enzymes. This time dependent approach revealed whether the fish could acclimate physiologically over time to suspended sediment exposure.

For the third chapter, I investigated the impact of suspended sediment on the zebrafish gut microbiome. Using the same fish from chapter two, I studied how suspended sediment (and potential recovery) alters the diversity and composition of the zebrafish gut microbiome. I used 16S rRNA gene sequencing on samples collected at one, three, seven and 28 days of exposure and recovery. By analyzing alpha and beta diversity metrics and identifying specific indicator species, I determined the extent and duration of the microbial community's response, providing crucial new insights into the sublethal effects of sediment on fish health.

CHAPTER ONE

Chronic, sublethal exposure to suspended sediment impacts gut function in *Danio rerio*

ABSTRACT

Anthropogenic activities increase suspended sediment in aquatic ecosystems, posing chronic physiological challenges for fish. This study investigated the effects of chronic (28 days) exposure to suspended kaolin clay (45 mg/L) on zebrafish (Danio rerio), hypothesizing that ingested sediment acts as a gut perturbation leading to reduced food consumption. We assessed somatic growth, whole-body cortisol, gill and gut histology, gill HSP60/HSP70 gene expression, and digestive enzyme activities. Sediment-exposed fish exhibited significantly shorter final body lengths and reduced relative gut lengths ($P < 0.05$ for both), supporting the perturbation hypothesis and suggesting decreased food intake. Histological analysis revealed increased goblet cells in the gut, indicating heightened mucus production. Alanyl-aminopeptidase activity, vital for protein digestion, was significantly reduced ($P < 0.05$). Conversely, whole-body cortisol and gill HSP expression showed no significant differences, suggesting acclimation. While preliminary due to pseudoreplication, these findings indicate that chronic suspended sediment acts as a gastrointestinal irritant, impairing nutrient assimilation. This highlights the critical need for further robust research to understand the ecological implications of sediment on fish foraging and digestion in impacted aquatic environments.

INTRODUCTION

Aquatic ecosystems (including riverine and coastal systems) are remarkable in that they provide tremendous value to humanity: ecosystem goods and services, such as import and export goods between states and nations, sources of food, and these habitats are used for recreation. Moreover aquatic ecosystems help with climate regulation, flood control, soil retention and nutrient cycling, to name a few (Costanza et al. 1997). In addition to aquatic ecosystems encompassing a rich biodiversity of plants and animals, they also are the home to a large (1.2

billion) and increasing number of people who live within 100 km of the coast (Nicholls and Small 2002) or along large river systems. As human communities adjacent to water continue to grow, mega cities (defined as having ten million inhabitants in an area) have also been on the rise. Amazingly, 67% of megacities are situated near large bodies of water, including rivers (von Glasow et al. 2013).

To maintain and support cities adjacent to waterways, development is necessary. Anthropogenic activities such as dredging, logging, and other alterations to waterways are part of these developments and act as stressors to aquatic ecosystems. For instance, dredging, the removal of sediment in shipping channels in marine and freshwater systems, creates sediment plumes in the water column, disturbs benthic communities, and destroys substrates vital for larval settlement. Logging removes plants that have roots to hold sediment in place, and without it, soil erosion increases at a faster rate, allowing loose soil to runoff into waterways during rain events or via human runoff (e.g., irrigation) (He et al. 2014). Another example of waterway alterations are creating pathways to drain excess water and its contents from rivers into near by lakes which are referred to as spillways. For example the largest river in North America, the Mississippi River, employs a spillway directed into Lake Pontchartrain. While excess water is diverted, so is suspended sediment. Modifications of water and land benefit us humans, however the effects need to be studied to understand the changes in the aquatic environments (Wenger et al. 2017).

Suspended sediment is composed of small particulates (organic matter, silt, clay, and sand) that can stay suspended in waterways causing the water column to become turbid and reduce light penetration, thus decreasing euphotic zone depth. This means fishes experience reduced visual acuity and behavioral avoidance of newly turbid waters (Collin and Hart 2015). Hence, visual predators are at a disadvantage of acquiring nutrients. In addition, since fish respire with gills in contact with the surrounding water, exposure to the suspended sediment can damage fish gill tissue, and alter the gill microbiome (Hess et al. 2015; 2017). The gill is essential for respiration, osmoregulation, pH regulation, nitrogen balance, and hormonal secretion (Evans et

al. 2005). Although the role of the gill microbiome in fish health is unknown, changes in it may impact fitness (Xiong et al. 2019). Thus, increased suspended sediment caused by human activity is a potential stressor for fishes, at least on a superficial level (Wenger et al. 2017).

Suspended sediment is known as a potential stressor that diminishes the quality of life for aquatic organisms (Kerr et al. 1995). Response measurements can range from lethal to sub-lethal (Newcombe and Macdonald 1991; Wenger et al. 2017). Behavioral effects of avoidance and reduced predator-prey activity are clearly documented (Utne-Palm 2002), but examples of sub-lethal physiological effects of suspended sediment are limited, particularly for chronic (≥28 days) levels of exposure. Of the physiological sub-lethal investigations, past studies have focused on gill physiology as it is vital to fish oxygen consumption (Wenger et al. 2017).

There are few studies of how suspended sediment impacts fish gut structure and function. For example, Dai et al. (2010) highlight how montmorillonite suspended in the water potentially protects Tilapia from lead accumulation. On the other hand, Nadermann et al. (2019) saw increased time to satiation in goldfish (*Carassius auratas*) exposed to turbid waters. Other studies suggest that suspended sediment is a vehicle for heavy metal and other toxins to enter into the digestive tract of fishes (Jayaprakash et al. 2015; Chen et al. 2022). Furthermore there is a growing literature on the ingestion of clay by terrestrial animals (including humans), both intentionally, or in animal feeds (Cranford and Gordon 1992; Urban and Kirchman 1992; Habold et al. 2009; Damato et al. 2022). The primary function of the gut is to extract nutrients from ingested food, but like the gill, the gut also engages in secretion and excretion and regulation of fish physiology, and the enteric microbiome engages with the fish immune system (Karasov and Douglas 2013; Egerton et al. 2018). Suspended sediment can reduce surfactant activity in the gut, which can play a role in how much nutrients can be absorbed in deposit feeding animals (Smoot and Findlay 2000), and ingested clay can impact lipid absorption in rats (Habold et al. 2009). If ingested sediment interferes with digestion and absorption, fishes may have problems meeting their nutritional demands. Although focused on gene expression, Nadermann et al.

(2019) found increased peptide YY expression (indicator of increased appetite), and longer feeding times in *C. auratas* exposed to suspended sediment, suggesting that it took longer to meet nutritional targets, thus leading to stimulation of the hunger center of the brain. Therefore, given the dearth of knowledge on how suspended sediment impacts fish physiology in general, and the gut in particular, more investigations of how suspended sediment impacts fish physiology are needed.

In this study, we exposed *D. rerio* to suspended sediment (kaolin clay) for 28 days to examine how they responded to prolonged exposure to suspended sediment. After the 28 day period, we assessed the following in the zebrafish exposed to the different systems: body size, gut length, whole body cortisol concentrations, gill and gut histology to observe how the structure of these tissues changed with clay exposure, Heat Shock Protein 70 (HSP70) and HSP60 expression in the gill (Yamashita et al. 2010b), and the activities of seven digestive enzymes in the gut that represent the ability of the fish to digest a suite nutrients (Table 1). We hypothesized that clay ingested with food could be experienced in one of two ways: 1) simply a diluent of potential nutrients, thus necessitating consumption of more food to meet nutrient requirements (similar to detritivorous fishes (e.g., (German 2009; Dai et al. 2010; Manna et al. 2020) or 2) a potential perturbation, that either made food harder to obtain, or necessitated consuming less food to avoid the potential deleterious impacts of ingested clay on gut structure and function (e.g., (Kolok et al. 1996; Nadermann et al. 2019). Overall, this is the most comprehensive examination of fish physiological response to suspended sediment to date and we hope it sheds light on how suspended sediment may impact fish.

MATERIALS

Sediment Exposure Experiment

Twenty-four *D. rerio* were obtained from and maintained at the University of California, Irvine, for many months before the experiment began. The experiment began with fish transferred into two re-circulating systems (n = 12 fish per system) with their own sump, biological, activated carbon,

and UV filtration. Fish were housed in groups of six in 10L tanks in each system. No particulate filtration was used on either system because we were adding suspended sediment to one system and particulate filters would remove the suspended clay. Deionized water with appropriate salts added was used in both systems. Temperature was maintained at 25°C, Ammonia and nitrogen concentrations along with pH and conductivity were monitored every day. Feces were siphoned out on demand and tanks were scrubbed once a week. Fish in both systems were fed Adult Zebrafish Complete Diet (Zeigler Brothers, Gardners, PA, USA) twice a day to satiation. This is the same food they were fed before the experiment began (Leigh et al. 2018). All procedures were engaged under approved Institutional Animal Care and Use Committee protocol AUP 21-012 (D.P. German, PI).

The difference between the two systems was that the sediment-exposed fish experienced water containing 45 mg/L (0.06 optical density units, ODU) sediment. Commercially available kaolin clay was used. A BioTek Synergy H1 Hybrid spectrophotometer/fluorometer equipped with a monochromator (BioTek, Winooski,VT, USA) was used to measure the optical density of the water in the tanks to confirm that the suspended sediment load was maintained at the same level across the 30-day experiment. Every other day, the net absorbance was measured and if it was less than 0.05 – 0.07 ODU, 3 grams of clay was added to the sump. If absorbance exceeded 0.07 ODU, ¼ of the sump was drained and replaced with fresh water until the desired optical density was achieved.

After twenty eight days, the experiment concluded. Eight fish from the control system and seven fish from the sediment system were sacrificed in buffered water with tricaine methanesulfonate (MS-222, Argent Chemicals Laboratory, Inc., Redmond, WA, USA). The fish were then measured (total length, mm), and dissected on a chilled cutting board. The digestive tracts were removed, measured, weighed, and frozen in liquid nitrogen and stored at -80°C until homogenized for digestive enzyme assays (Leigh et al. 2018). The right and left gills were removed for qpcr and histology, respectively. The right gills were fixed in RNAlater (Thermo-

Fisher), and the left gill and additional fish intestines were placed in Bouin's fixative. The eviscerated bodies were frozen in liquid nitrogen and stored at −80°C until they were homogenized for cortisol measurements.

Assays, Cortisol and Digestive enzymes

For the cortisol assay, each frozen body was homogenized in a 25 mM Tris-HCl buffer (pH 7.5), with the buffer volume determined by the mass of the tissue. The homogenization was performed using a Polytron homogenizer at 3,000 rpm for two 30-second intervals. The resulting homogenate was then centrifuged at 9,400 g for two minutes at four degrees celsius. A cortisol assay was then performed on the supernatant using an ELISA kit, following the manufacturer's protocol. For the enzyme assays, individual frozen digestive tracts were diluted with a 25 mM Tris-HCl buffer (pH 7.5) based on mass and then homogenized according to the methods of German and Bittong (2009). The enzyme assays were conducted in duplicate or triplicate at 25°C using a BioTek Synergy H1 Hybrid spectrophotometer/fluorometer, as described by German and Bittong (2009).

Amylase activity was measured following the Somogyi-Nelson method (Nelson 1944; Somogyi 1952). Briefly, 10 μL of intestinal homogenate was combined with 90 μL of starch substrate (1%), and the amount of glucose released after 30 minutes was determined using a glucose standard curve and expressed in U (µmol glucose liberated per minute) per gram wet weight of gut tissue.

Maltase activity was measured following the glucose oxidase-peroxidase method of Dahlqvist (1968). Using a glucose standard curve, maltase activity was expressed in U (µmol glucose liberated per minute) per gram wet weight of gut tissue.

Trypsin activity was measured following the Erlanger et al. (1961) method, using a p-nitroaniline standard curve, and expressed in U (µmol p-nitroaniline liberated per minute) per gram wet weight of gut tissue.

Lipase activity was measured following Iijima et al. (1998). Using a p-nitrophenol standard curve, activity was expressed in U (µmol p-nitrophenol liberated per minute) per gram wet weight of gut tissue.

Aminopeptidase activity was measured following Roncari and Zuber (1969). Additionally, using a p-nitroaniline standard curve, activity was expressed in U (µmol p-nitroaniline liberated per minute) per gram wet weight of gut tissue.

Alkaline phosphatase activity was measured following German and Bittong (2009), using 200 μM of 4-methylumbelliferyl-phosphate dissolved in 25 mM Tris-HCl (pH 7.5). Briefly, 90 μL of substrate were combined with 10 μL of homogenate in a black microplate and incubated for 30 minutes. Following incubation, 2.5 μL of 1 M NaOH was added to each microplate well, and the fluorescence read immediately at 365 nm excitation and 450 nm emission. Each plate included a standard curve of the product (4-methylumbelliferone, MUB), substrate controls, and homogenate controls, and enzymatic activity (nmol product released per minute per gram wet weight tissue) was calculated from the MUB standard curve.

Histological Analysis

After 48 hours in Bouin's fixative, gill and gut tissues were removed from the fixative and rinsed in 0.1 M phosphate buffered saline (PBS), pH 7.5, 3 times for 60 min, and a final rinse overnight at 4°C. Following rinsing in PBS, the tissues were rinsed for 60 min in running DI water and dehydrated in a graded ethanol series. The tissues were paraffin embedded, sectioned, and stained in hematoxylin-eosin stain by Veranex (Boston, MA, USA).

RNA and qPCR

Total RNA from gill samples were isolated using TRIzol reagent (Thermo Fisher Scientific) following the manufacturer's protocol. Then, using BioTek Synergy H1 Hybrid spectrophotometer, total RNA concentration and quality was measured. Using iScript™ cDNA Synthesis Kit (Bio Rad USA), the RNA was converted to cDNA using reverse transcriptase. 1 µl of the resulting cDNA was used in a 1× SYBR Green mix (Bio Rad USA) and amplified with a thermal cycler. Primer

sequences for target HSP-70 were 5′-3′ GAA AGG CAA GAT CAG CGA GG and CCC TCC CTG GTA GAG TTT GG. Primer sequences for HSP-60 were 5′-3′ GTC GGC GAG GTC ATT GTT AC and GCC AGT CGT TCG TTG AGT TT for reference Beta-Actin were 5′-3′ CGA GCT GTC TTC CCA TCC A and TCA CCA ACG TAG CTG TCT TTC TG. Expression was measured with the Δ-ΔCt method (Livak and Schmittgen 2001).

Statistical Analysis

Prior to all significance tests, a Bartlett's and Shapiro-Wilk test for equal variance and normality were performed on all data sets. All tests were performed using R studio. If passed two sample t-test were ran for initial body length, gut length and relative gut length. If data did not pass, a Boxcox transformation was performed. If data, it did not pass a Wilcoxon signed-rank test was performed.

RESULTS

The fish in the two treatments looked different, with the fish exposed to sediment having a lighter color, perhaps due to the white clay being in the mucus on the fish body (Fig. 1). The initial mass and length of zebrafish exposed to suspended sediment and ambient tank water did not differ significantly (mass, t = 1.707, df = 19.24, P= 0.104, length, t = 1.085, df = 22, P = 0.2896). Similarly, there were no significant differences in final mass between the two treatments (mass, t = 1.404, df = 12.56, P = 0.184). However, a significant difference was observed in final length, with zebrafish in the suspended sediment treatment exhibiting a shorter final length (length t = 2.454, df = 8.405, P = 0.038).

The relative gut lengths were significantly reduced in zebrafish exposed to suspended sediment in comparison to those not exposed to sediment (t = 6.219, df = 12, P < 0.001; Fig. 2). Whole body cortisol levels, however, were not significantly different between the two treatments (control treatment concentration = 6207.596 ng/g, suspended sediment treatment = 6168.768 ng/g) (t = 0.024, df = 13, P = 0.981,). The analysis of gene expression showed no statistically

significant changes for either *hsp70* or *hsp60*. The expression of *hsp70* showed a 4.61 fold change (P = 0.275), and the expression of *hsp60* showed a 4.26 fold change (P = 0.235). Qualitative analysis of gill histology suggested less organized gill filaments in fish exposed to suspended sediment, whereas gut histology suggested that control fish exhibited less lipid and goblet cells compared to fish exposed to suspended sediment (Fig. 3).

The digestive enzyme activity data revealed that most enzymatic activities did not differ, except for alanyl-aminopeptidase, which was significantly reduced in the fish exposed to suspended sediment (Table 2). Mean alkaline phosphatase activity in the fish exposed to suspended sediment was more than double that in the control fish, but the variability was too high to detect any statistical differences (Table 2).

DISCUSSION

In this study, we tested what happens to zebrafish when they are exposed to a sublethal concentration of suspended sediment for an extended period of time (28 days), that would be considered chronic exposure. With our data, we tested two hypotheses: the dilution hypothesis, which states that suspended sediment ingested with food may act as a diluent, thus meaning the fish have to consume more food to meet their nutritional demands, or a perturbation hypothesis, which states that ingested sediment is a gut irritant, which would lead to lower consumption of food by fish exposed to suspended sediment (Table 1). The latter would come with growth consequences, as animals eating less would not grow as quickly. Although our results were mixed, we largely found support for the perturbation hypothesis, as the sediment-exposed fish were of shorter lengths at the end of the experiment, had shorter relative gut lengths (Fig. 2), which suggests that they ate less across the experiment, had more goblet cells in gut tissue cross section (suggesting more mucus production; Fig. 3), and significantly lower alanyl-aminopeptidase activities in their guts (Table 2), suggesting compromised protein digestion and

absorption in comparison to those fish not exposed to suspended sediment. Other parameters did not vary significantly, but we will discuss each below.

The digestive system is metabolically costly to operate (Cant et al. 1996), and thus, it must be modulated to match the content and quantity of material passing through the alimentary canal (Karasov and Rio 2020). Modulation can include changes in gut size (especially length), enzymatic activity, and enteric microbial diversity (Leigh et al. 2018; Herrera et al. 2022; Barts et al. 2025; Kohl et al. 2025). Indeed, all of these traits have changed with diet in zebrafish (Leigh et al. 2018; 2022). In this study we found a significant reduction in relative gut length of zebrafish exposed to suspended sediment. The shorter gut implies that, despite being offered the same amount of food as the control fish, the sediment-exposed fish ate less, and therefore, their guts got smaller, perhaps to conserve resources. This finding supports the hypothesis that suspended sediment is a perturbation and the fish may eat less to avoid it, or just have a harder time locating food due to impaired visual ability. Interestingly, goldfish reared in turbid environments took longer to consume a similar amount of food in comparison to those raised without suspended sediment, and thus, the sediment-exposed fish had higher peptide YY expression in their guts, suggesting a larger appetite (Nadermann et al. 2019). Thus, we conclude that suspended sediment can impact food intake in animals that do not normally ingest sediment, such as those not living in benthic environments.

Digestion is a series of chemical reactions and digestive enzymes are the catalysts. Therefore, digestive enzymatic activities are typically measured as a metric of digestive capability (Karasov and Douglas 2013). Modulation of digestive enzyme activities in fishes fed different diets is well studied. Generally, some species show flexibility (Leigh et al. 2018), whereas others may show less (German et al. 2004). With a reduction in intake due to sediment exposure, we might expect a reduction in enzymatic activities in sediment-exposed zebrafish since there are less nutrients streaming through the gut per unit time. Interestingly, the only enzyme we found to decrease in activity in response to sediment exposure was alanyl aminopeptidase, which supports

past findings of this enzyme being plastic (Le et al. 2023; Rankins et al. 2023). This enzyme generates absorbable amino acids from dipeptides, and therefore, a reduction in activity implies reduced capacity to absorb amino acids from their diets. However, the endoprotease that digests polypeptides, trypsin, did not change in activity in response to sediment exposure. Thus, the only pathway that we observed to be impacted by sediment exposure is amino acid absorption, and this may be a significant problem that fishes encounter following exposure to suspended sediment.

Although alkaline phosphatase activity was not significantly different among the fish in the different treatments, it was highly variable in the sediment-exposed fish. This enzyme is involved in controlling gut inflammation, dephosphorylation of lipopolysaccharide (LPS) and protection against lipopolysaccharide toxicity (Bates et al. 2007; Lallès 2010; 2020) . This variability could reflect individual differences in microbial colonization, subtle environmental factors, or even genetic predispositions. For instance, fluctuations in specific bacterial populations known to produce or be affected by LPS might lead to varying demands on alkaline phosphatase activity (Lallès 2010). The observed variability in alkaline phosphatase activity might reflect a dynamic interplay between the host and the gut microbiota in regulating lipid metabolism and inflammation. Future studies with larger sample sizes and additional analyses, such as correlating individual alkaline phosphatase activity levels with specific microbial taxa and inflammatory markers, are needed to elucidate the precise role of alkaline phosphatase in maintaining gut homeostasis in this model. Furthermore, targeted interventions to manipulate alkaline phosphatase activity (Lallès 2020) could provide valuable insights into its functional significance.

A link between the intestinal microbiome and exposure to suspended sediment may also be evident with the increased goblet cells in the epithelium of the sediment-exposed fish (Fig. 3). Increased goblet cell number implies that the fish may be generating more mucus to protect the epithelial lining, which in turn can impact the microbial community along the epithelium (Yao et al. 2024). Future work should explore the impacts of sediment ingestion on the enteric microbiome.

The experimental design in this study has limitations. Specifically, the use of a single recirculating system for each treatment group (control and sediment), with fish distributed across two tanks within each system, inherently restricts our statistical power to discern tank-specific effects. Consequently, 'tank' could not be treated as an independent variable in our analyses, resulting in a pseudoreplicated design with an effective sample size of N=1 per treatment (Hurlbert 1984). Furthermore, our methodology involved collective measurements of initial and final length and mass, precluding the analysis of individual growth trajectories. While we can assess differences in overall size between treatment groups at the conclusion of the experiment, detailed insights into individual growth rates would be better. These limitations necessitate cautious interpretation of our findings and underscore the need for future investigations employing experimental designs with true biological replicates, such as the multi-system approach. Nevertheless, this initial investigation provides novel evidence suggesting potential alterations within the gut environment with ingestion of suspended sediment, warranting further exploration into the underlying mechanisms and their ecological implications.

In terms of growth, we see that both treatments had no significant difference in mass before and after, but the suspended sediment treatment was significantly shorter in length post sediment exposure. The shorter lengths in the sediment group supports the perturbation hypothesis, but the lack of data supporting that the fish were “stressed” (i.e., no changes in whole body cortisol or gill HSP70 expression) suggests that this level of suspended sediment doesn’t necessarily stress the fish out on a twenty eight -day scale.

Cortisol plays a central role in mediating the stress response through the activation of the hypothalamic-pituitary-interrenal (HPI) axis, which is the fish equivalent of the mammalian hypothalamic-pituitary-adrenal (HPA) axis (Shaughnessy et al. 2023). Prolonged or chronic stress can lead to adjustments in this system. For instance, Madaro et al. (2015) demonstrated that Atlantic salmon exposed to chronic stress in the form of chasing for three weeks subsequently exhibited a blunted plasma cortisol response when faced with an additional novel stressor of being

netted exposed to air and confined in a bucket. This finding supports the broader understanding that the HPI axis can become downregulated or acclimated during chronic stress, indicative of stress acclimation (Barton and Schreck 1987; Madaro et al. 2015). Furthermore, Atlantic parr fish, over 23 days, saw a big difference in how much the fish grew. The control fish got 41% bigger, but the fish under chronic stress 8.5% bigger, and had a suppression of appetite (Madaro et al. 2015). Both results support that stress can affect body size at level of ingestion and digestion efficiency.

In the context of our study, it is plausible that continuous exposure to suspended sediment over the four-week period may have initially triggered an increase in cortisol levels. However, as the exposure persisted, cortisol levels might have subsequently decreased due to HPI axis downregulation or acclimation to the chronic stressor. To better understand the physiological response to chronic suspended sediment, future research should consider sampling whole-body cortisol at multiple time points throughout the exposure, or measuring plasma cortisol in a time series (which is difficult in animals as small as zebrafish). A time series could provide a clearer picture of the temporal dynamics of cortisol regulation under such conditions.

Heat shock proteins (HSPs) are highly conserved proteins crucial for maintaining cellular balance and providing general protection in all living things (Yamashita et al. 2010). Organisms produce these HSPs when they encounter stressful conditions (Parsell and Lindquist 1993; Hofmann 1999) . For example, Himalayan fish gill tissue showed a gradual increase in HSP60 expression, and an initial spike in HSP70 expression, over time under chronic thermal stress (Kaur et al. 2025). Here, we only examined the possibility of chronic stress after a 28 day exposure to suspended sediment, which did not produce changes in HSP60 or HSP70 gene expression. However, measuring HSP60 and HSP70 levels across multiple time points in our experiment could have revealed whether either gene experienced a transient upregulation at the onset of suspended sediment exposure or a sustained change throughout the duration. It's important to consider that zebrafish, being naturally riverine fish, are inherently exposed to suspended

sediment in their flowing water habitats. Consequently, this species may not exhibit a typical stress response to suspended sediment in the same way it might to other environmental challenges.

In conclusion, although limited by design, our study suggests that a 28 day exposure to suspended sediment may pose some challenges for the gill and gut of zebrafish. Thus, this leads to a call for more detailed studies of sediment exposure over time to examine whether a sudden onset of sediment (e.g., from dredging or precipitation) could pose a risk as a potential acute stressor, or whether it is limited to being more of a problem as a chronic one. A design with better replication and the ability to track individuals will be crucial to testing the perturbation hypothesis further. Nonetheless, ingested sediment may, at the very least, make it harder to find and ingest food, which should be accounted for when assessing potential risks of sediment plumes in multiple environments (Wenger et al. 2017).

Table1: Predicted gut physiological responses of zebrafish under two hypotheses tested in this study involving fish exposed to suspended sediment *vs* those that were not: Dilution Hypothesis (Increased Feeding in response to sediment) and Perturbation Hypothesis (Irritation/Reduced Feeding in response to sediment).

Physiological Parameter	**Dilution Hypothesis (Increased Feeding relative to control)**	**Perturbation Hypothesis (Reduced Feeding relative to control)**
Feeding behavior	Increased consumption (compensating for lower nutrient density)	Decreased consumption (due to sediment irritation)
Gut length	Longer	Shorter
Gut tissue integrity	Increased	Decreased
Digestive enzyme activity	Elevated	Lower
Whole body cortisol levels	Low	Higher

Table 2. Digestive enzyme activities in zebrafish exposed to suspended sediment and those that were not exposed (control).

Enzyme Activity (µmol min^{-1} g^{-1})	**Treatment**	**Mean**	**Statistic**
Amylase activity	Control	12.393±6.271	*t* = 0.80654, df = 13, *P* = 0.4344 *t*-test
	Suspended Sediment	14.509±3.132	
Maltase activity	Control	117.6±51.613	*t* = 0.097983, df = 10, p-value = 0.923 *t*-test
	Suspended Sediment	119.925±31.0363	
Trypsin activity	Control	2.290±1.333	*t* = 0.54951, df = 10, p-value = 0.5947 *t*-test
	Suspended Sediment	1.910±1.067	
Lipase activity	Control	0.975±0.359	W = 29, p-value = 0.284 Wilcoxon Rank Sum Continuity Correction
	Suspended Sediment	0.938±0.979	
Aminopeptidase activity	Control	0.596 ±0.279	t = 3.3014, df = 11, p-value < 0.005 *t*-test
	Suspended Sediment	0.286±0.055	
Alkaline Phosphatase (AP) activity	Control	351.745±54.96	W = 15, p-value = 0.432 Wilcoxon Rank Sum Continuity Correction
	Suspended Sediment	729.868±552.052	
N-acetyl-β-D-glucosaminidase (NAG) activity	Control	220.889±137.3805	*t* = 1.7279, df = 10, p- value = 0.1147 *t*-test
	Suspended Sediment	538.795±389.826	

Note: The mean enzyme activity (µmol min^{-1} g^{-1}) is reported for each treatment group as mean ± standard error. Activities for AP and NAG are in nmol min^{-1} g^{-1}. Statistical comparisons between control and suspended sediment groups were performed using t-tests or Wilcoxon Rank Sum tests, as indicated. Significant differences ($p < 0.05$) are bolded.

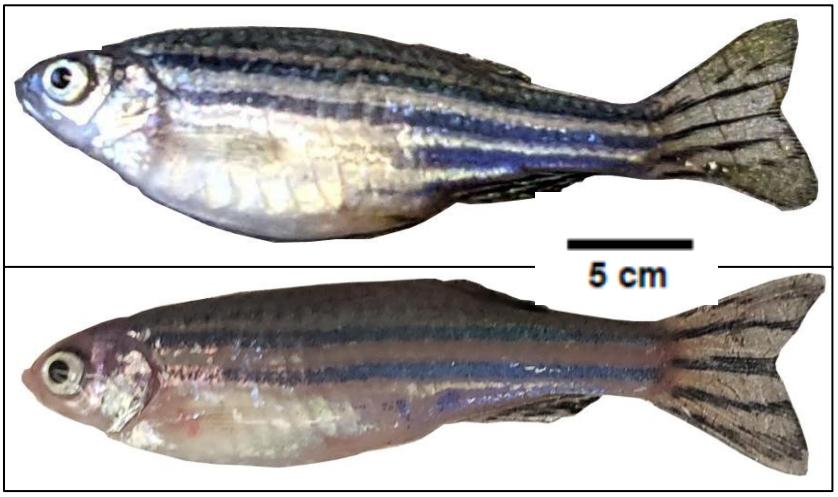

Figure 1. Zebrafish specimens showing the effects of different treatments. The top image depicts a zebrafish from the control treatment, displaying normal coloration and appearance. The bottom image shows a zebrafish from the suspended sediment treatment. Note the apparent presence of sediment particles embedded within the mucus of the fish, indicating the impact of sediment exposure.

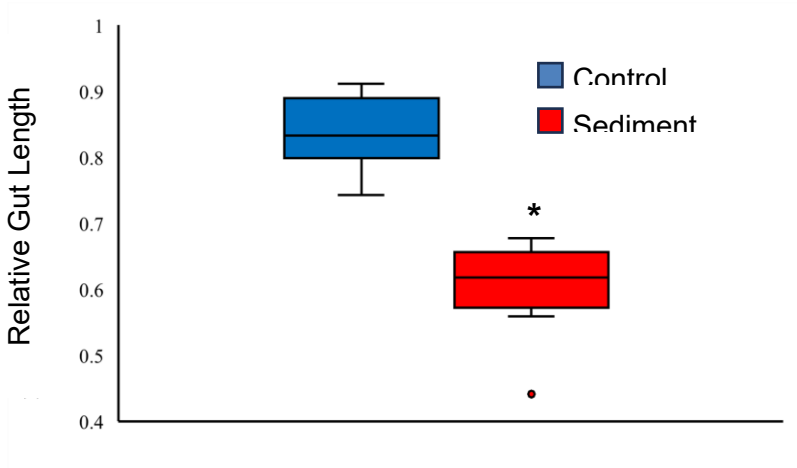

Figure 2. Box plot of relative gut length (gut length (mm)/standard length (mm)) in zebrafish exposed to suspended sediment, or to control conditions with no sediment. Relative gut lengths were compared with *t*-test, showing that the sediment exposed fish had shorter relative gut lengths than the control fish ($t = 6.2192$, df = 12, $P < 0.001$), indicated with the asterisk.

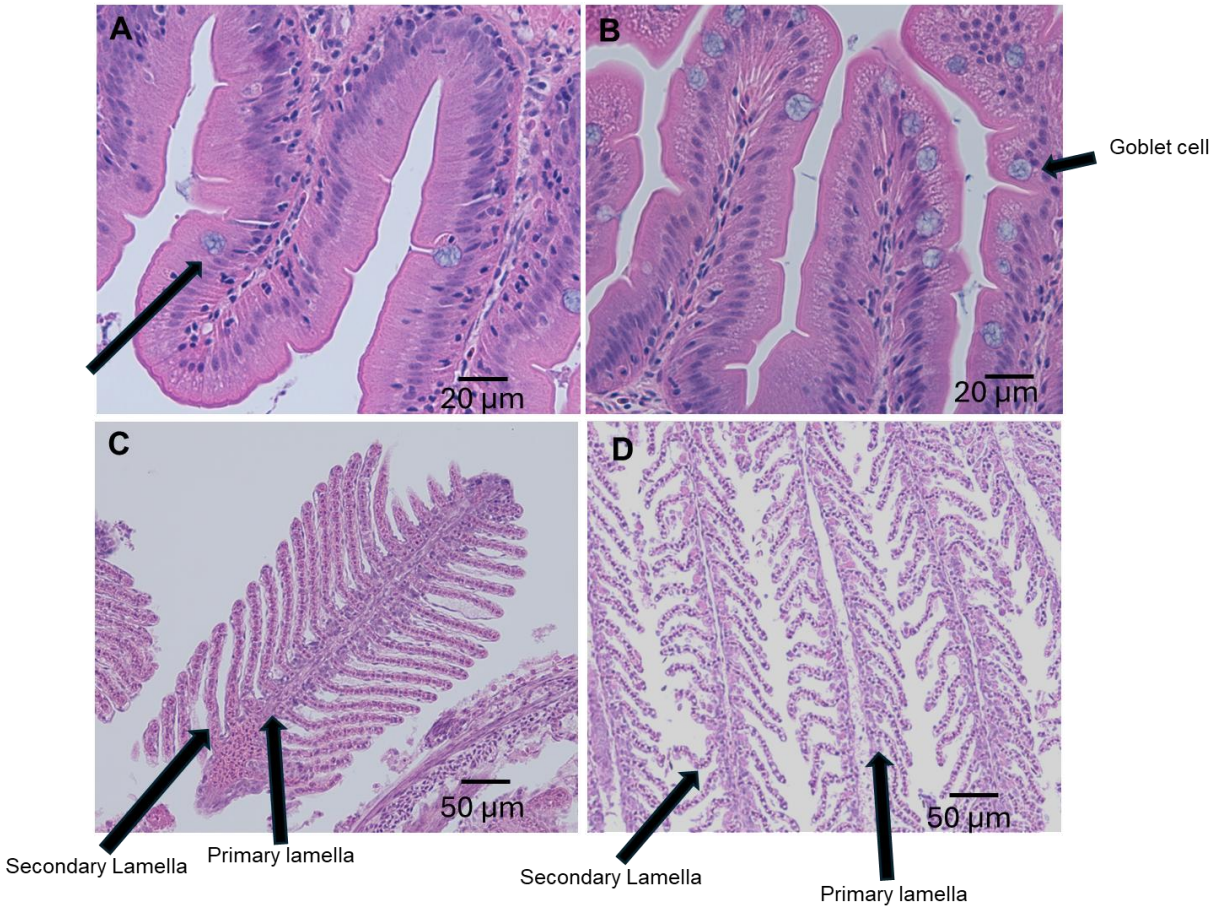

Figure 3. Histological images of fish tissues under control and suspended sediment treatments. (A) Gill tissue from the control treatment. (B) Gill tissue filaments are less organized from the suspended sediment treatment. (C) Proximal intestine tissue from the control treatment. (D) Proximal intestine tissue from the suspended sediment treatment showed more goblet cells. All images taken at 20x magnification.

CHAPTER TWO

Acute and chronic, sublethal exposure to suspended sediment impacts gut function and recovery in *Danio rerio*

ABSTRACT

Suspended sediment is a widespread environmental stressor, yet the long-term physiological costs and the potential for recovery in fish are not well understood. This study characterized the time-course of the physiological response of zebrafish (Danio rerio) to chronic sediment exposure and their capacity for recovery. Fish were exposed to suspended kaolin clay (45 mg/L) for one, three, seven, and 28 days, with parallel recovery groups returned to clear water. We assessed gill heat shock protein (*hsp60* and *hsp70*) gene expression and the activity of several digestive enzymes. With significant changes in HSP expression only observed at early time points (one and seven days), indicating physiological acclimation. While sediment exposure had no significant effect on fish mass or length, it impacted digestive physiology. Over 28 days, control fish exhibited a significant increase in amylase, maltase, and alkaline phosphatase activity. This increase over time was completely absent in both the sediment exposed and recovery groups, resulting in significantly lower enzyme activity compared to controls by day 28. Protein digestion was compromised with longer exposure, and some enzyme activity levels of the recovery group did not return to control levels. These findings suggest that fish acclimate to chronic sediment exposure through a physiological trade-off that suppresses normal enzymatic changes that occur over time. The lack of improvement in the recovery group indicates that this is not an immediately reversible condition, highlighting the potential for transient pollution events to have lasting sublethal impacts on fish health.

INTRODUCTION

Anthropogenic activities such as dredging and land development are increasing the concentration of suspended sediment in aquatic ecosystems, posing a significant challenge to the health of resident fishes (Wenger et al. 2017; Chapter one). While the effects of turbidity on

visual predators and gill health are documented (Collin and Hart 2015; Hess et al. 2015), the sublethal physiological consequences of chronic exposure, particularly concerning gut function, remain less understood. The gut is a critical site for nutrient assimilation, and any interference with its function can have cascading effects on fish growth, health, and fitness (Karasov and Douglas 2013).

In a previous investigation (see Chapter one), we exposed zebrafish (*Danio rerio*) to suspended sediment for 28 days and found evidence supporting a "perturbation hypothesis", where ingested sediment acts as a gut irritant. Fish in that study exhibited reduced final body lengths, shorter relative gut lengths, and dampened aminopeptidase activities, suggesting potential compromise of protein digestion with sediment ingestion. It appeared that the fish ate less to avoid the negative impacts of the ingested clay. However, that initial study was limited by a pseudoreplicated design and a single endpoint measurement, which precluded an understanding of the temporal dynamics of a response to suspended sediment. It remained unclear whether the observed effects were the result of an acute, unacclimated response or a sustained, chronic condition. Investigating such a difference is quite nuanced. For instance an acute response to a perturbation can occur anywhere between an hour to 72 hours (Newcombe and Jensen 1996; Isono et al. 1998; Berli et al. 2014), while a chronic alteration can range from 10 to 30 days (Newcombe and Macdonald 1991; Wenger et al. 2014; Madaro et al. 2015). For example, we know suspended sediment can drive plasticity and increase size of the eyes and brain in African cichlids when reared in turbid waters (Tiarks et al. 2024). Additionally swimming performance was detrimentally affected in salmonids due to acute (less than 24 hours) suspended sediment exposure (Berli et al. 2014). Even with these very interesting studies, one variable the literature has not fully resolved is how the duration of exposure mediates the transition from an acute to a chronic response. Consequently, we lack a clear understanding of a fish's capacity to acclimate over time versus suffering from sustained physiological perturbation due to suspended sediment exposure.

To address these knowledge gaps, the current study was designed to characterize the physiological response to suspended sediment across time and to assess the potential for recovery. We exposed zebrafish to suspended kaolin clay for one, three, seven, and 28 days and included a recovery group that was transferred from water containing suspended sediment to sediment-free water for a period equal to their exposure (Fig. 1). We measured a suite of physiological parameters, including body size, gut length, digestive enzyme activities and gill heat shock protein (*hsp60* and *hsp70*) gene expression. Gut length provides information on food intake, whereas digestive enzyme activities provide information on the fish's capability to digest dietary substrates (Karasov and Douglas 2013). HSP60 and HSP70 are part of the vertebrate stress response (IWAMA et al. 1999; Basu et al. 2002). We hypothesized that the initial exposure (one to three days) would trigger an acute stress response, which would diminish over time as the fish acclimated to the sediment exposure. Such a response would be similar to how fish acclimate to temperature changes (Berli et al. 2014; Fortin-Hamel and Chapman 2024). Furthermore, we predicted that the negative impacts on digestive physiology would be reversible, with enzyme activities in the recovery group returning to levels observed in fish never exposed to suspended sediment (control animals). This study provides a more comprehensive examination of the sublethal impacts of suspended sediment, offering critical insight into the capacity for fish to acclimate to and recover from this widespread environmental perturbation.

METHODS

Experimental Design and Animal Husbandry

This study utilized adult zebrafish as a model organism to assess the physiological effects of suspended sediment exposure and subsequent recovery. The experiment involved four different exposure durations (one, three, seven, and 28 days), with fish divided into three treatment groups: control, suspended sediment exposed, and recovery. Each treatment condition consisted of twelve fish housed in three replicate one-liter tanks (N=4 fish per tank; Fig. 1). All

tanks were part of a larger re-circulating system that included biological, activated carbon, and ultraviolet filtration but lacked particulate filtration, since this would remove the suspended sediment from that system. The control groups were kept in clear, sediment free water, while the suspended sediment exposed groups were maintained in a constant suspension of kaolin clay at 45 mg/L. The control and sediment exposure systems were on the same shelf, in the same room, just centimeters apart, but had different sump systems. They received water from the same reservoir, but the sediment system had kaolin clay added. The recovery group fish were first exposed to the same sediment concentration for a set duration and then that tank was cleaned (fish were placed in a holding tank containing aquarium water for 10 minutes during cleaning) and transferred to the control system for an equal period of time before sampling. See Chapter One for details on how the suspended sediment was maintained in the experimental system.

Sample Collection and Processing

Adult zebrafish that were sourced and maintained at the University of California, Irvine, were individually tagged with a subcutaneous visible implant elastomer (Northwest Marine Technology, Inc., Anacortes,WA) for unique identification. Using a three milliliter syringe, .29 gauge, 0.3 cc insulin, fish were first anesthetized then tagged either near the dorsal fin or tail. After tagging, fish were allowed to recover for at least 24 hours before the experiment commenced. Following their duration in the experiment, fish were sacrificed in buffered water with 1% tricaine methanesulfonate (MS-222, Argent Chemicals Laboratory, Inc., Redmond, WA, USA). The fish were then measured (total length, mm), and dissected on a chilled cutting board. The digestive tracts were removed, measured, weighed, and frozen in liquid nitrogen and stored at -80°C until homogenized for digestive enzyme assays (Leigh et al. 2018). The right and left gills were removed for qPCR and histology, respectively. The right gills were fixed in RNAlater (Thermo-Fisher), and the left gills were placed in Bouin's fixative. Additional fish intestines were placed in McDowell and Trump fixative (McDowell and Trump 1976). All frozen samples were

stored at -80°C until further processing. All procedures were engaged under approved Institutional Animal Care and Use Committee protocol AUP 21-012 (D.P. German, PI).

Enzyme Assays

The activities of six digestive enzymes (amylase, maltase, trypsin, aminopeptidase, lipase, and alkaline phosphatase) were quantified from digestive tract homogenates, as described in Chapter One. The homogenization was performed using a Polytron homogenizer at 3,000 rpm for three 30-second intervals at a speed of 7-8. About 1200 microliters of 25mM Tris-HCl Tris(hydroxymethyl)aminomethane hydrochloride buffer with a pH of 7.5 was used to dilute the gut while homogenizing (dilution factor~ 50). The resulting homogenate was then centrifuged at 9,400 g for two minutes at four degrees celsius, and the supernatant was stored in 100 μL aliquots in a -80°C freezer until used in enzyme assays.

Gene Expression Analysis (qPCR)

Total RNA from gill samples were isolated using TRIzol reagent (Thermo Fisher Scientific) following the manufacturer's protocol. Then, using a BioTek Synergy H1 Hybrid spectrophotometer, total RNA concentration and quality was measured. Using iScript™ cDNA Synthesis Kit (Bio Rad USA), the RNA was converted to cDNA using reverse transcriptase. One µl of the resulting cDNA was used in a one × SYBR Green mix (Bio Rad USA) and amplified with a thermal cycler (Chan et al. 2023). Primer sequences for target *hsp70* were 5′-3′ GAA AGG CAA GAT CAG CGA GG and CCC TCC CTG GTA GAG TTT GG. Primer sequences for *hsp60* were 5′-3′ GTC GGC GAG GTC ATT GTT AC and GCC AGT CGT TCG TTG AGT TT (Xie et al. 2015) for reference Beta-Actin were 5′-3′ CGA GCT GTC TTC CCA TCC A and TCA CCA ACG TAG CTG TCT TTC TG (Tiedke et al. 2013).

Statistical Analysis

All statistical analyses were performed using R version 4.3.1. A significance level of $P<0.05$ was used for all statistical tests. For all analyses, the tank in which a given animal was housed was taken into account and showed no significance. For enzyme activity analyses, the effects of treatment and duration on the activity of four enzymes (amylase, maltase, lipase,aminopeptidase and alkaline phosphatase) were assessed using a two-way Analysis of Variance (ANOVA). The model for each enzyme included treatment, duration, and the treatment-by-duration interaction as fixed effects. Prior to analysis, the assumptions of normality of residuals and homogeneity of variance were tested for each model using the Shapiro-Wilk and studentized Breusch-Pagan tests, respectively. When a significant interaction effect was detected, pairwise comparisons were conducted using the emmeans package with a Tukey adjustment for multiple comparisons. To determine the statistical significance of changes in gene expression, a non-parametric bootstrapping approach was employed (Chan et al. 2023). For each time point (one, three, seven, and 28 days), the sediment-exposed and recovery treatment groups were independently compared against their respective time-matched control group. The mean ΔCt for each group was bootstrapped with 5,000 replicates to generate a distribution of the difference in means (ΔΔCt) (Livak and Schmittgen 2001). A two-sided p-value was calculated from this distribution to test the null hypothesis of no difference between the treatment and control groups.

RESULTS

Morphological Variables

The effects of treatment and duration on fish morphology were assessed using Two-Way ANOVAs and ANCOVAs, with statistical assumptions validated for each model. For post exposure mass, after a Box-Cox transformation and correction for heteroscedasticity, an ANCOVA revealed no significant effects of treatment or time. In contrast, post exposure length was significantly influenced by duration ($F_{3, 130} = 4.19$, $P = 0.007$), with a marginally significant treatment by duration interaction ($F_{6, 130} = 2.06$, $P = 0.062$, Fig. 2). A consistent and strong treatment by duration

interaction was observed for both gut length ($F_{6, 128}$ = 3.91, *P* = 0.001, Fig. 4) and relative gut length (RGL) ($F_{6, 130}$ = 3.54, *P* = 0.003, Fig 4). This interaction was primarily driven by the response of the sediment group at Day 3. At this time point, sediment-exposed fish exhibited significantly shorter guts compared to the recovery group (*P* = 0.011) and a significantly lower RGL compared to both control (*P* = 0.050) and recovery (*P* = 0.008) groups. Furthermore, within the sediment group, both gut length and RGL at Day 3 were significantly lower than at all other time points (*P* ≤ 0.001) (Fig. 5).

Gill HSP Gene Expression Analysis

Fold change indicates the magnitude of difference in gene expression between an experimental sample and a control sample, normalized to a housekeeping gene. A fold change greater than 1 signifies upregulation, while a value less than 1 indicates downregulation (Goni et al. 2009). At the one day time point, the expression of *hsp70* in the sediment exposed group was significantly up regulated compared to the control (fold change = 0.47, *P* = 0.05). The recovery group also showed a significant downregulation compared to the control (fold change = 0.37, *P*= 0.02) of *hsp70* expression. The change in *hsp60* expression in the sediment exposed group was not statistically significant (Fold Change = 0.99, *P*= 0.828). However in the recovery group *hsp60* expression was significantly down regulated compared to the control (fold change = 0.27, *P* < 0.001, Fig. 6).

After 3 days of suspended sediment exposure, there were no significant differences for *hsp70* expression (fold change = 2.14, *P*=0.526). Additionally, for the recovery group there was no significant change in the *hsp70* expression (fold change=2.88 *P*=0.396). There was no significant change in the *hsp60* expression for the sediment and recovery treatment (fold change=1.06, *P*=0.850; fold change=1.26, *P*=0.898, Fig 6).

After one week, the expression of *hsp70* in the sediment group showed a fold change of 2.10 and was not statistically significant from the control (*P*= 0.816). For the recovery group, there

was no significant change with a fold change of 1.69 (P = 0.380). For *hsp60* expression the sediment group had a significant upregulation with a fold change of 3.20 (P=0.042). The recovery group had no significant change (fold change= 1.86, P=0.64), Fig 6.

At the 28-day time point, no significant changes in gene expression were observed. For the sediment group, the fold change for *hsp70* was 2.15 (P = 0.314). For the recovery group, the fold change for *hsp70* was 3.92 (P = 0.100) and for *hsp60* was 1.85 (P = 0.379) and the sediment group had a fold change of 1.87 (P=0.386, Fig. 6).

Enzyme Activity Results

Amylase

A two-way ANOVA on amylase activity revealed a significant interaction between treatment and duration ($F_{6, 68}$ = 2.25, P = 0.049). Comparisons between treatments at each time point revealed no significant differences at days one, three, or seven. At day 28, however, amylase activity was significantly higher in the control group compared to the recovery group (P = 0.017). An analysis of changes over time showed that within the control group, activity at day 28 was significantly higher than at day one (P = 0.009) (fig. 4) and day three (P = 0.011). No significant changes over time were observed within the sediment or recovery groups Fig. 7)

Maltase

A two-way ANOVA on maltase activity revealed a non-significant interaction between treatment and duration ($F_{6, 72}$ = 1.98, P = 0.080). Exploratory comparisons showed that at day 28, maltase activity was significantly higher in the control group compared to the recovery group (P = 0.034). Within the control group, activity at day 28 was significantly higher than at day one (P = 0.042) (Fig. 8). For analysis within each treatment and across time there was no significant difference.

Trypsin

A Box-Cox transformation was applied to the trypsin data. The two-way ANOVA on the transformed data revealed significant main effects of treatment (P = 0.015) and duration (P = 0.031), as well as a significant interaction between treatment and duration ($F_{6, 71}$ = 2.96, P = 0.012). At day three, trypsin activity was significantly lower in the control group compared to the sediment group (P < 0.001). An analysis of changes over time showed that within the control group, activity was significantly higher at day one compared to day three (P = 0.023). Within the sediment group, activity was significantly higher at day one and day three compared to day 28 (P = 0.012 and P = 0.045, respectively) (Fig. 9).

Aminopeptidase

A non-parametric Scheirer-Ray-Hare test was performed on aminopeptidase activity and revealed significant main effects of both treatment (H = 7.72, P = 0.021) and duration (H = 12.79, P = 0.005). Follow-up Kruskal-Wallis tests showed a significant difference among sediment and recovery treatments at day 28 (P = 0.0009). Within the suspended sediment group, the one day treatment was significantly different from the three day (P = 0.21), seven day (P < 0.001), and 28 day treatments (P < 0.001; Fig. 10).

Lipase

A non-parametric Scheirer-Ray-Hare test was performed on lipase activity and revealed a highly significant interaction between treatment and duration (H = 33.30, P < 0.001). Follow-up Kruskal-Wallis tests showed significant differences among treatments at day one between sediment and recovery groups (P = 0.002), day three between sediment and recovery groups (P = 0.046), and day 28 between sediment and control (P = 0.0471) and sediment and recovery groups (P = 0.030), with sediment-exposed fish having lower lipase activity than the other groups (Fig. 11). There was no significant effect of duration across time for the control fish. There was a significant effect of time for sediment in which one day was significantly different from the rest of

the durations (*P*=0.0211) and for the recovery group day one was significantly different from day three and day 28 (*P*= 0.0071; Fig. 11).

Alkaline Phosphatase

A Box-Cox transformation was applied to the alkaline phosphatase data. The two-way ANOVA on the transformed data revealed a significant main effect of duration ($F_{3, 73}$ = 2.85, *P* = 0.043) and a non-significant interaction (*P* = 0.055). An analysis of changes over time within the control group showed that activity at day 28 was significantly higher than at day one (P = 0.009) and day three (P < 0.001). No significant changes over time were detected in the sediment or recovery groups (Fig. 12).

DISCUSSION

In this study, we tested how zebrafish respond to a sublethal concentration of suspended sediment and whether time in sediment free water would allow them to return to their non-exposed condition. Our prior work (Chapter One) suggested that ingested sediment acts as a gut perturbation, leading to reduced growth and compromised protein digestion. The current, more robust experimental design allows us to refine this conclusion. While our new results do not show the same impacts on fish growth, they point toward a more subtle physiological trade-off. Our results suggest that zebrafish may acclimate to chronic sediment exposure, but this acclimation could come at the cost of suppressing normal physiological changes in their digestive systems.

A significant difference was observed in body length post exposure between the control and sediment treatments at the one day time point. A significant difference was also noted within the sediment group over time, between the one and three day time points. This suggests that the observed variation in body length within the sediment group is not solely a function of treatment or time, but may be due to natural variation among individual fish. With regard to body mass, there were no significant differences observed between treatments or across time, indicating that the masses of all fish were similar and remained consistent throughout the experiment.

The digestive system is metabolically costly to operate and is modulated to match the quantity and quality of ingested material (Karasov and Martínez del Rio 2007). We also see how the digestive tract is able to modulate in size (Duque-Correa et al. 2024). We see a decrease in RGL at day three. This may signify a transient negative effect of sediment exposure on digestive tract morphology (Dane and Şişman 2020), that could involve changes in enterocyte cell size (Leigh et al. 2018). After day three however, the sediment treatment RGL increased in size and was not significantly different from the other treatments at each time point. This again demonstrates acclimation to the environment and further evidence supporting why there was not a significant difference in RGL between the control and recovery (Yuen et al. 2007).

Zebrafish are naturally omnivorous (Ulloa et al. 2011; Leigh et al. 2018). Thus they need a variety of enzymes to help break down their ingested food. Of those enzymes, trypsin is one that is vital to break down proteins into smaller peptides (Solovyev et al. 2023). Our results show a decrease in trypsin activity at the 28 day mark within the sediment treatment, signifying that suspended sediment may behave as a perturbation to the fish (Chapter One). In both experiments, protein digestion (aminopeptidase activities in Chapter One, trypsin and aminopeptidase activities in this study) appeared to be compromised with longer sediment exposure. This finding supports the perturbation hypothesis in that this reduced activity may imply either reduced ability to digest or reduced digestion overall. In a study in which Songpu mirror carp experienced starvation stress for one, two, three, and four weeks, the four week treatment group experienced reduced general protease activity (Zhao et al. 2022). Moreover, the loricariid catfish, *Pterygoplichthys disjunctivus*, fed a low-quality diet over several months had reduced trypsin activity, suggesting that reduced proteolytic capability indicates compromised digestion (German et al. 2010). Lipase, an enzyme that breaks down fats was significantly lower within the sediment group for days three, seven and 28 compared to the one day. Similar to protease activity, Zhao et al. (2022) also observed a dampening of lipase activity with starvation stress. Overall, compromised protein and lipid digestion observed in this study show how vital enzymes

are affected by disturbances like suspended sediment. This has significant ecological implications, as it suggests that organisms exposed to transient suspended sediment events may carry a physiological cost that could impair their fitness later in life, particularly if they subsequently face other challenges such as food scarcity or competition.

Another enzyme of interest was alkaline phosphatase, which is involved in controlling gut inflammation, dephosphorylation of lipopolysaccharide (LPS) and protection against lipopolysaccharide toxicity (Bates et al. 2007; Lallès 2010; 2020). In Chapter One, its activity was variable in the sediment-exposed fish, inferring some type of disruption in its function and also influencing an immune response, however between treatments, this enzyme was not significantly different in this experiment. This is an important finding because it rules out the question of variability triggering an immune response via alkaline phosphatase that was alluded to in the Chapter One.

Control fish exhibited a significant increase in amylase, maltase, and alkaline phosphatase activity by day 28, a physiological change over the course of the experiment that did not happen in the other treatments, thus making an examination of activities over time in each group of great importance. The increases in enzyme activity, specifically in the control group at the 28 day duration, were not related to the technician running the assays or day of measurement, suggesting it is a real increase with unknown underpinnings. The fact that so many enzymes were elevated on that day remains unexplained, but reinforces not relying on the comparison among groups at specific durations, but across time.

A novel and critical component of this study was the inclusion of a recovery group. The finding that some enzyme activity profiles of the recovery fish were indistinguishable from the continuously exposed fish implies that any changes in digestive enzymatic changes may not be a readily reversible state. This reinforces the concept of the ability of digestive enzymes to modulate (Olsson and Holmgren 2001). In addition this supports that the digestive system is responsive to changes in the gut (Gheisvandi et al. 2015)

The concept of acclimation to a perturbation is clarified by the temporal data in this experiment. Here, we observed significant changes in *hsp70* and *hsp60* expression only at the one day and seven day time points. This pattern of an acute, transient stress response followed by a return to baseline levels is consistent with physiological acclimation to a chronic stressor (Barton 2002). It is plausible that continuous exposure to suspended sediment initially triggers cellular stress pathways, but as the exposure persists, these systems may downregulate as the fish acclimates. The lack of a significant HSP response at the 28-day mark (Chapter One and this investigation) could therefore be interpreted not as an indication of no effect, but rather as a potential sign of acclimation (Methling et al. 2010; Kaur et al. 2025).

In conclusion, this study refines our understanding of the sublethal impacts of suspended sediment on fish. While our first experiment identified sediment as a gut perturbation, this work sheds light on a potential mechanism of acclimation. It appears that zebrafish can acclimate to chronic sediment exposure without immediate impacts on growth, but they do show some dampening of digestive system function, specifically relating to protein and lipid digestion. This apparent energetic trade-off leads to a physiological state that is less equipped for efficient nutrient assimilation, a deficit that persists even when suspended sediment is not present within the water column for some enzymes. These data suggest that suspended sediment is something that impacts fish physiology, and that recovery is necessary (and possible on some levels, but not others) before fish are exposed to more sediment. Thus, things like dredging should be ceased following rainfall events that are already introducing sediment to the waterways via runoff, to allow fish to be prepared for further exposure to suspended sediment.

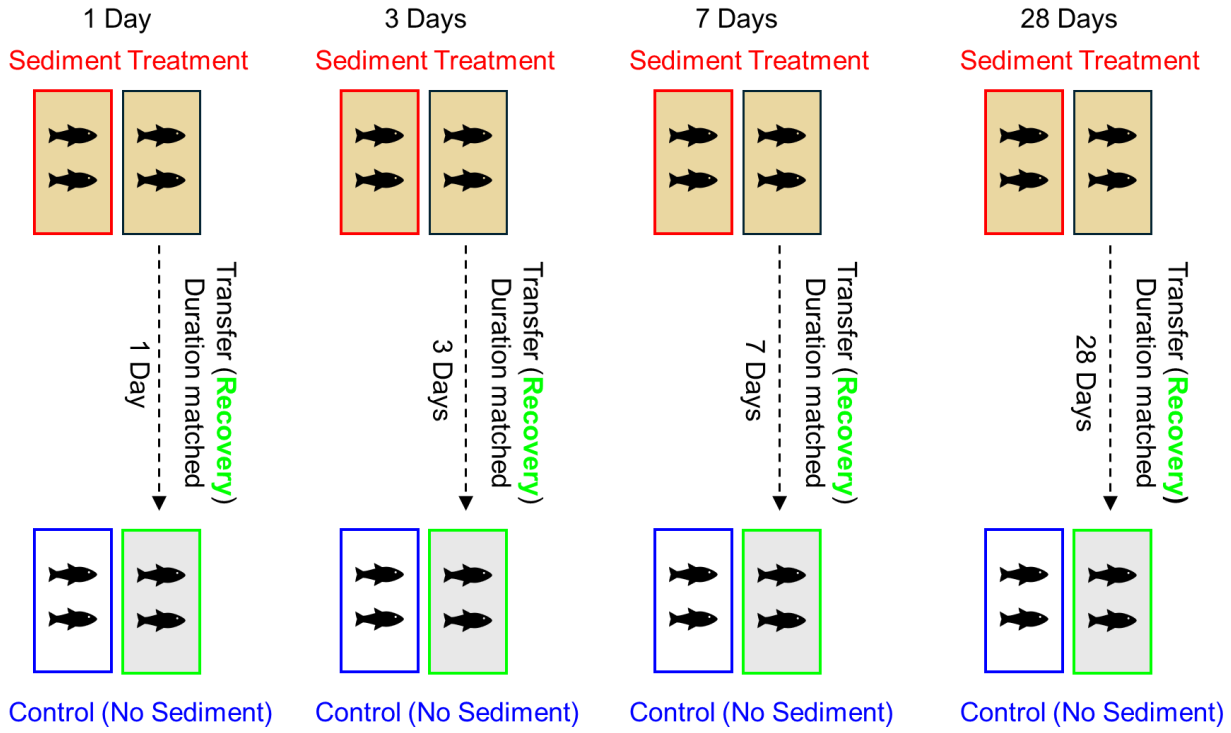

Figure 1. Experimental design of the treatment (control, sediment and recovery), through the various durations of time.

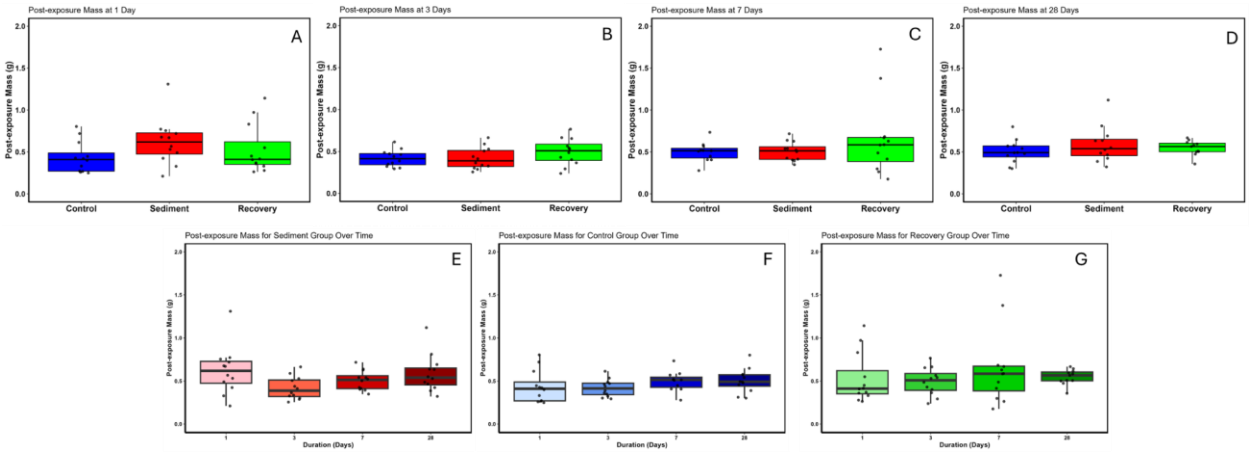

Figure 2. Panels A through D illustrate the distribution of post-exposure mass for each treatment group (control, sediment, and recovery) at specific time points: (A) 1 day, (B) 3 days, (C) 7 days, and (D) 28 days. Panels E through G show the change in post-exposure mass over time (1, 3, 7, and 28 days) for each of the three treatment groups: (E) control, (F) sediment, and (G) recovery. Statistical significance between treatments is denoted by different letters (e.g., 'a' and 'b').

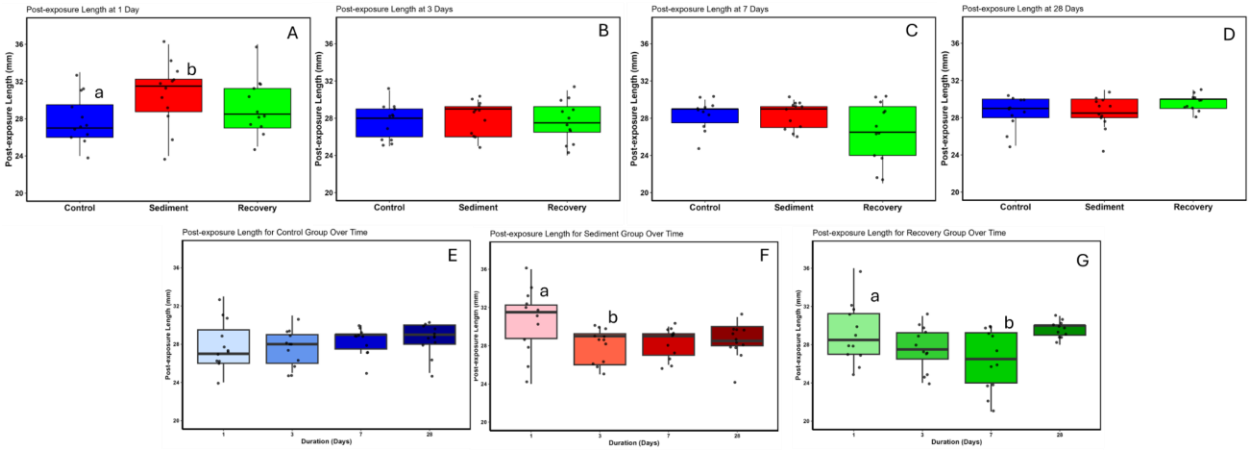

Figure 3. Panels A through D illustrate the distribution of post-exposure length for each treatment group (control, sediment, and recovery) at specific time points: (A) 1 day, (B) 3 days, (C) 7 days, and (D) 28 days (P = 0.007). Panels E through G show the change in post-exposure length over time (1, 3, 7, and 28 days) for each of the three treatment groups: (E) control, (F) sediment, and (G) recovery (P = 0.062). Statistical significance between treatments is denoted by different letters (e.g., 'a' and 'b').

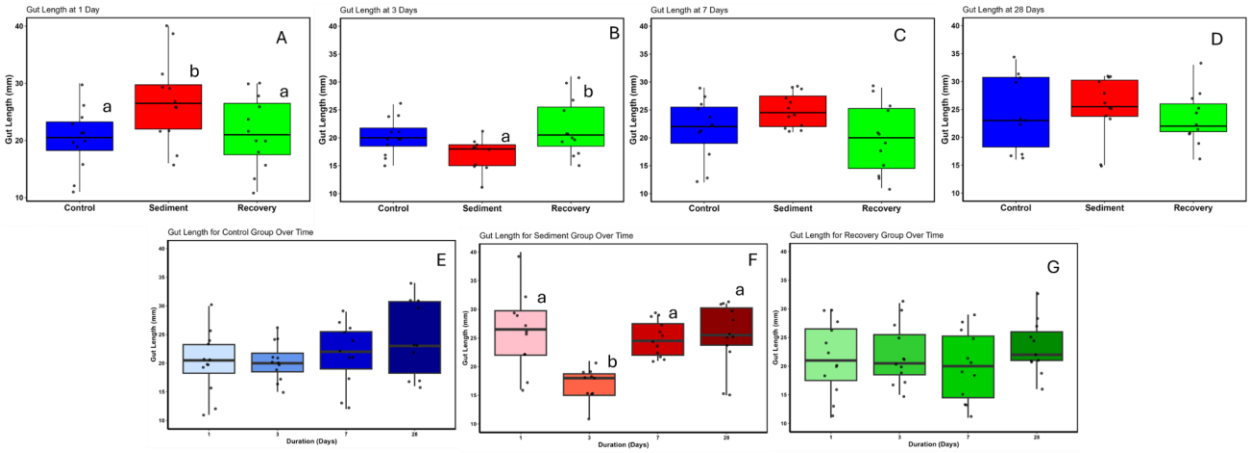

Figure 4. Panels A through D illustrate the distribution of gut length for each treatment group (control, sediment, and recovery) at specific time points: (A) 1 day, (B) 3 days, (C) 7 days, and (D) 28 days (P = 0.001). Panels E through G show the change in gut length over time (1, 3, 7, and 28 days) for each of the three treatment groups: (E) control, (F) sediment, and (G) recovery. Statistical significance between treatments is denoted by different letters (e.g., 'a' and 'b').

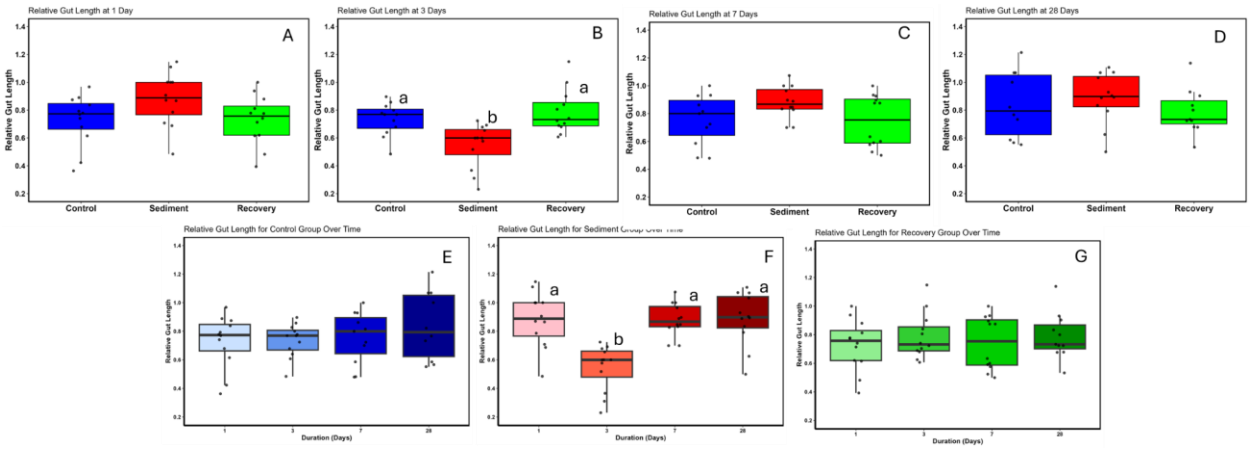

Figure 5. Panels A through D illustrate the distribution of RGL for each treatment group (control, sediment, and recovery) at specific time points: (A) 1 day, (B) 3 days, (C) 7 days, and (D) 28 days (P = 0.003). Panels E through G show the change in RGL over time (1, 3, 7, and 28 days) for each of the three treatment groups: (E) control, (F) sediment, and (G) recovery. Statistical significance between treatments is denoted by different letters (e.g., 'a' and 'b').

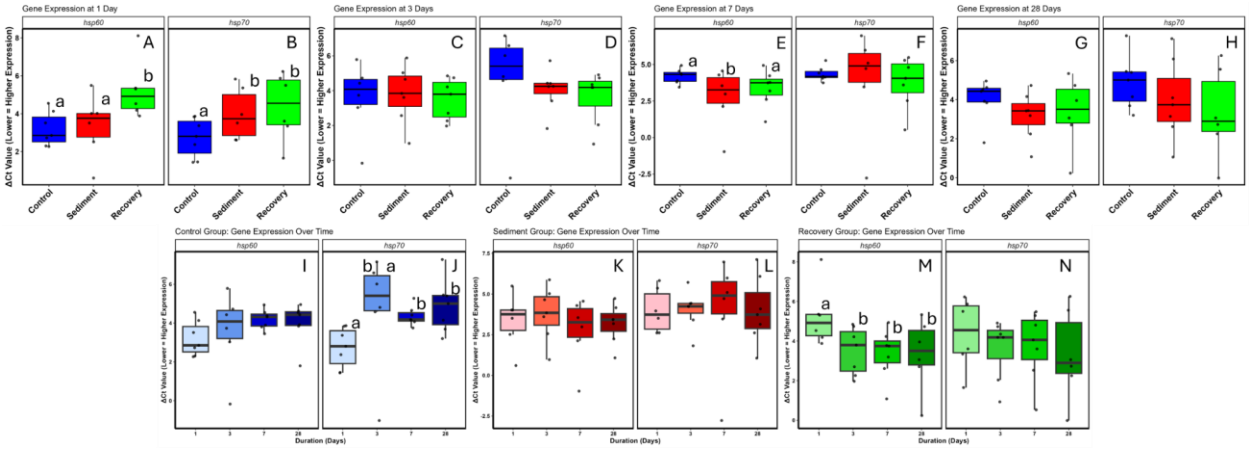

Figure 6. Gene expression of Heat Shock Protein 60 (*hsp60*) and Heat Shock Protein 70 (*hsp70*). Panels A-H show the expression levels at 1, 3, 7, and 28 days for the control, sediment, and recovery groups. Panels I and J show the changes in gene expression in the control group over time. Panels K and L show the changes in the sediment group over time, and Panels M and N show the changes in the recovery group over time. The y-axis represents the relative gene expression level calculated as ΔCt value, where a lower value indicates higher gene expression. Statistical significance between treatments is denoted by different letters (e.g., 'a' and 'b').At the one-day time point, the expression of hsp70 in the sediment exposed group was significantly down-regulated (P=0.05). The recovery group also showed a significant downregulation of hsp70 expression (P=0.02). The change in hsp60 expression in the Recovery

group showed a significant downregulation (P<0.001). The sediment group did have a significant upregulation of hsp60 (P=0.042).

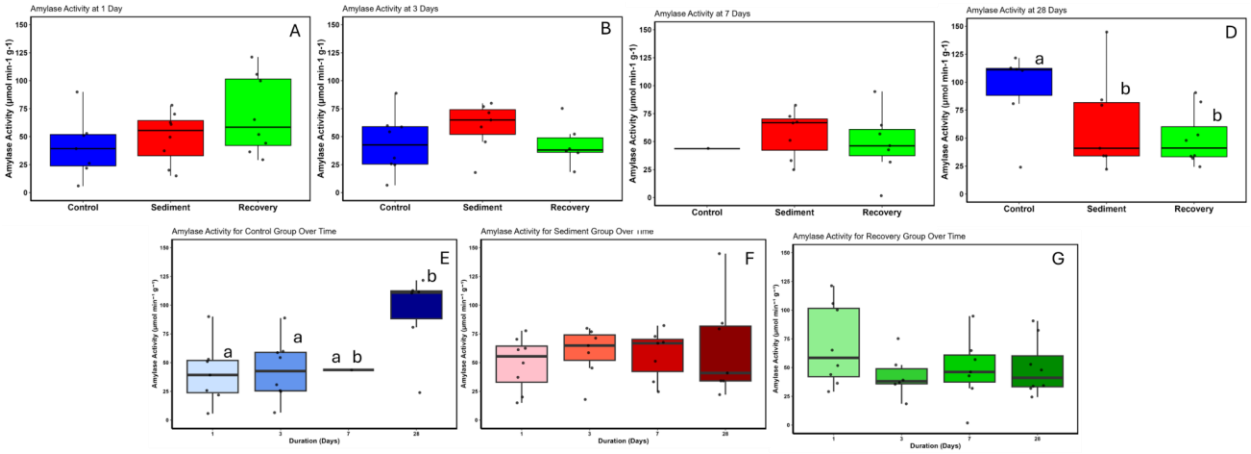

Figure 7. Box plots showing amylase activity under various experimental conditions. Panels A through D illustrate the distribution of amylase activity for each treatment group (control, sediment, and recovery) at specific time points: (A) 1 day, (B) 3 days, (C) 7 days, and (D) 28 days. Panels E through G show the change in maltase activity over time (1, 3, 7, and 28 days) for each of the three treatment groups: (E) control, (F) sediment, and (G) recovery (Table 2).

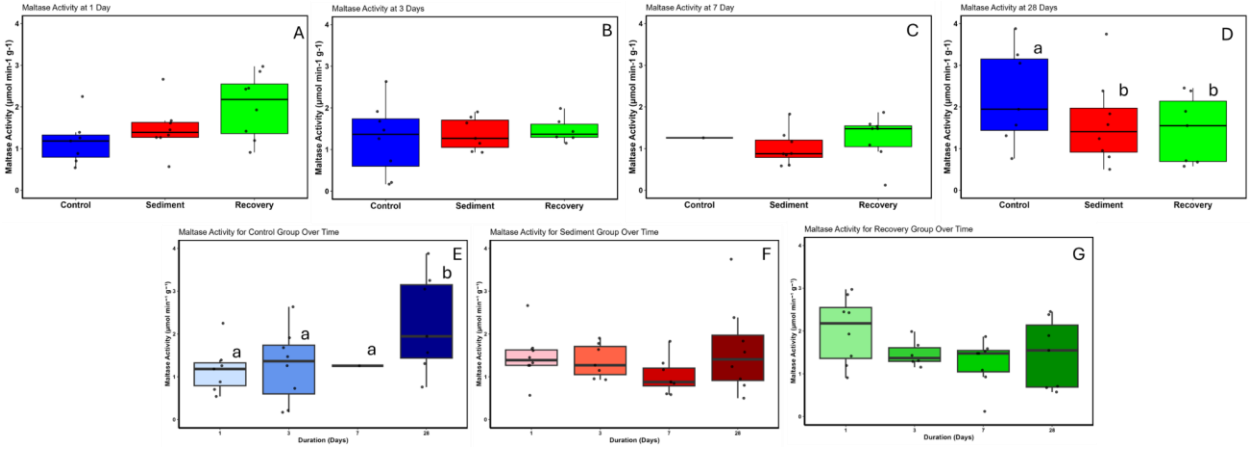

Figure 8. Box plots showing maltase activity under various experimental conditions. Panels A through D illustrate the distribution of maltase activity for each treatment group (control, sediment, and recovery) at specific time points: (A) 1 day, (B) 3 days, (C) 7 days, and (D) 28 days. Panels E through G show the change in maltase activity over time (1, 3, 7, and 28 days) for each of the three treatment groups: (E) control, (F) sediment, and (G) recovery (Table 2).

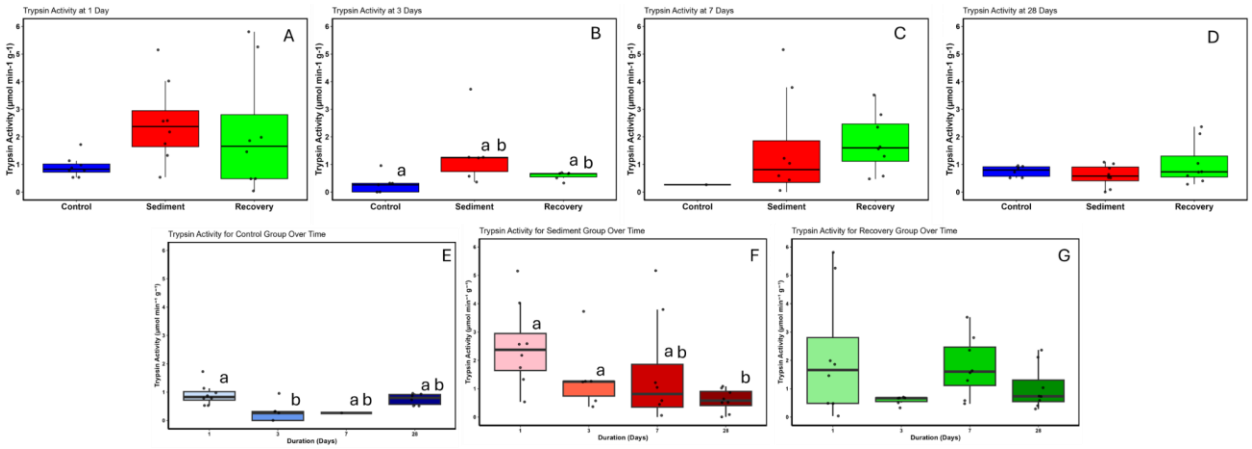

Figure 9 . Box plots showing trypsin activity under various experimental conditions. Panels A through D illustrate the distribution of trypsin activity for each treatment group (control, sediment, and recovery) at specific time points: (A) 1 day, (B) 3 days, (C) 7 days, and (D) 28 days. Panels E through G show the change in trypsin activity over time (1, 3, 7, and 28 days) for each of the three treatment groups: (E) control, (F) sediment, and (G) recovery (Table 2).

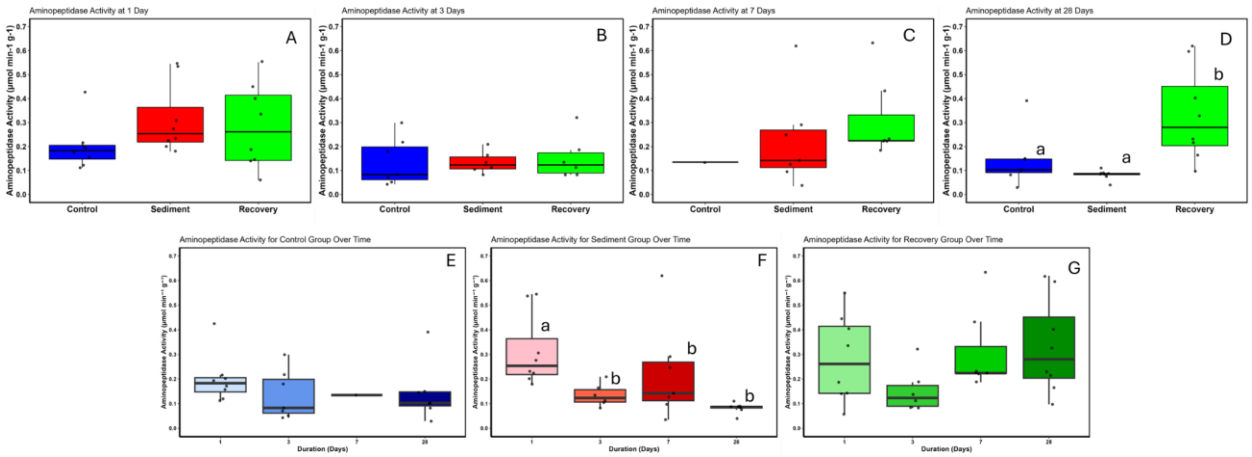

Figure 10. Box plots showing aminopeptidase activity under various experimental conditions. Panels A through D illustrate the distribution of aminopeptidase activity for each treatment group (control, sediment, and recovery) at specific time points: (A) 1 day, (B) 3 days, (C) 7 days, and (D) 28 days. Panels E through G show the change in aminopeptidase activity over time (1, 3, 7, and 28 days) for each of the three treatment groups: (E) control, (F) sediment, and (G) recovery (Table 2).

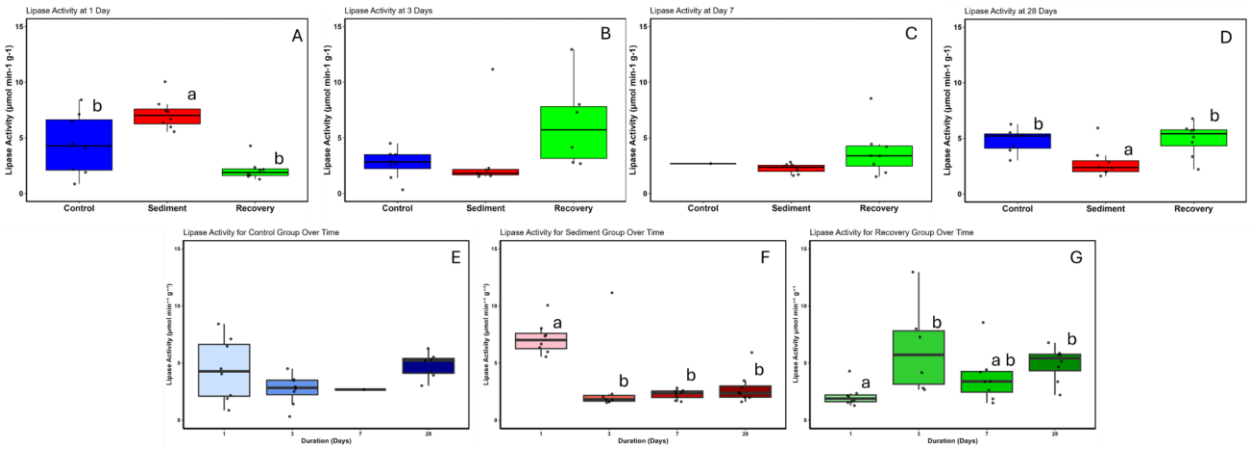

Figure 11. Box plots showing lipase activity under various experimental conditions. Panels A through D illustrate the distribution of lipase activity for each treatment group (control, sediment, and recovery) at specific time points: (A) 1 day, (B) 3 days, (C) 7 days, and (D) 28 days. Panels E through G show the change in lipase activity over time (1, 3, 7, and 28 days) for each of the three treatment groups: (E) control, (F) sediment, and (G) recovery (Table 2).

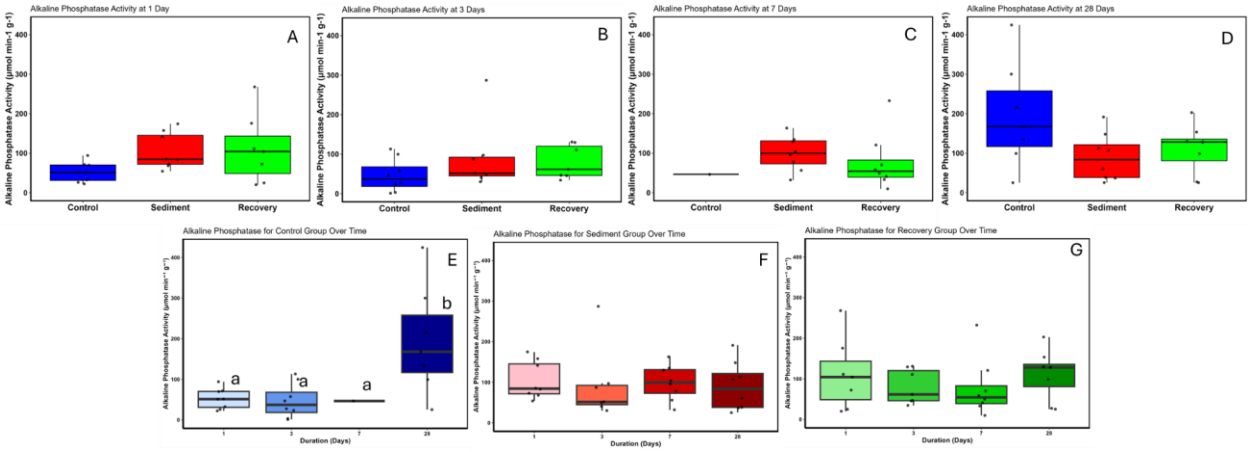

Figure 12. Box plots showing alkaline phosphatase activity under various experimental conditions. Panels A through D illustrate the distribution of alkaline phosphatase activity for each treatment group (control, sediment, and recovery) at specific time points: (A) 1 day, (B) 3 days, (C) 7 days, and (D) 28 days. Panels E through G show the change in alkaline phosphatase activity over time (1, 3, 7, and 28 days) for each of the three treatment groups: (E) control, (F) sediment, and (G) recovery (Table 2).

Table 1. Summary of statistical models testing the effects of treatment and duration on fish morphological variables. Response variables were transformed as needed to meet model assumptions. The table presents the overall model significance and the significance of individual factors and their interaction, showing F-statistics, degrees of freedom (Df), and P-values. Significance was determined at $P < 0.05$.

Response Variable	Model Type	Significant Interaction (Treatment x Duration)	Individual Effects Significance
Post Mass (log transformed)	Linear Model	Not Significant, $F_{5, 136} = 1.39$, $P = 0.232$	Treatment: Not Significant, Duration: Not Significant
Post Length	Generalized Linear Model (GLM) with Gamma error and log link	Not Significant Chi-square test, Df = 5, $P = 0.2015$	Treatment: Not Significant, Duration: Not Significant
Gut Length (Box-Cox transformed)	Linear Model	Not Significant, $F_{5, 134}$ $P = 1.67$, P = 0.146	Treatment: Not Significant, Duration: Significant
Relative Gut Length	ANOVA	Significant, $F_{6, 130} = 3.54$ $P = 0.003$ Day 3: Control > Sediment, $P = 0.04$ Recovery > Sediment, $P = 0.008$. Within Sediment: Day 1 > Day 3 $P = 0.0001$, Day 7 > Day 3 $P = 0.0003$, Day 28 > Day 3 $P = 0.0002$	Treatment: Not Significant, Duration Not Significant

Table 2. Summary of statistical analyses on digestive enzyme activities in response to treatment and duration. Results from Two-way ANOVAs or the non-parametric Scheirer-Ray-Hare test are shown. The table presents test statistics (F or H), degrees of freedom (Df), and P-values for main effects and interactions. Key significant findings from post-hoc tests are summarized. Significance was set at $P < 0.05$.

Enzyme	Statistical Test	Significant Interaction (Treatment x Duration)	Significant Main Effects	Key Findings from Follow-up Analysis
Amylase	Two-way ANOVA	Yes, $F_{6, 68}$ = 2.25, *P* = 0.049	No	Day 28: Control > Recovery, *P* = 0.017. Within Control: Day 28 > Day 1 *P* = 0.009 & Day 28 > Day 3 *P* = 0.011.
Maltase	Two-way ANOVA	No, $F_{6, 72}$ = 1.98, *P* = 0.080	No	Day 28: Control > Recovery *P* = 0.034. Within Control: Day 28 > Day 1 *P* = 0.042.
Lipase	Scheirer-Ray-Hare	Yes, H = 33.30, *P* < 0.001	No	Significant differences among treatments at Day 1 *P* = 0.002, Day 3 P = 0.046), and Day 28 *P* = 0.030.
Alkaline Phosphatase	Two-way ANOVA (Box-Cox transformed)	No, *P* = 0.055	Duration: Yes, $F_{3, 73}$ = 2.85, *P* = 0.043	Within Control: Day 28 > Day 1 P = 0.009) & Day 28 > Day 3 *P* < 0.001.
Aminopeptidase	Scheirer-Ray-Hare	No, *P* = 0.067	Treatment: Yes (H = 7.72, P = 0.021) Duration: Yes (H = 12.79, *P* = 0.005)	Significant difference among treatments at Day 28 *P* < 0.0009. Recovery > Control & Sediment. Within Sediment Day 1 > Day 3 *P* = 0.003, Day 7 *P* = 0.008, Day 28 0.021
Trypsin	Two-way ANOVA	Yes, $F_{6, 71}$ = 2.96, *P* = 0.012	Treatment: Yes, *P* = 0.015 Duration: Yes, *P* = 0.031	Day 3: Control < Sediment *P* < 0.001. Within Control: Day 1 > Day 3 *P* = 0.023. Within Sediment: Day 1 > Day 28 *P* = 0.012 & Day 3 > Day 28 *P* = 0.045.

CHAPTER THREE

Acute and chronic, sublethal exposure to suspended sediment impacts gut microbiome and recovery in Danio rerio

ABSTRACT

Anthropogenic activities are increasing suspended sediment in aquatic ecosystems, yet the effects on the fish gut microbiome are not well understood. This study investigated how the gut microbiome of adult zebrafish (Danio rerio) responds to different durations of suspended sediment exposure and subsequent recovery. Fish were exposed to suspended kaolin clay (45 mg/L) for one, three, seven, and 28 days, with parallel recovery groups returned to clear water. The gut microbiome was characterized using 16S rRNA gene sequencing. Results showed that suspended sediment caused an acute disruption to the gut microbial community. Alpha diversity (Shannon index) was significantly reduced in sediment-exposed and/or recovery groups at one and three days but not at the 28 day chronic time point. The diversity of the recovery group took seven days to become statistically indistinguishable from the control group. While overall community composition (beta diversity) did not show significant clustering, indicator species analysis revealed distinct microbial signatures for each treatment group. The control group hosted the highest number of significant indicator species, suggesting a stable baseline community that was altered by the sediment perturbation. These findings indicate that suspended sediment induces a rapid, but recoverable, loss of gut microbial diversity, and that even after diversity is restored, the underlying community composition remains altered.

INTRODUCTION

The gut microbiome is a community assemblage of microorganisms that inhabit the gut that are crucial for host health (Butt and Volkoff 2019; Herrera and German 2024). The microbes in the alimentary canal aid in digestion, nutrient absorption, and modulating the immune system (Llewellyn et al. 2014). Broadly, there are three factors that can influence the gut microbiome: the environment, diet, and the host (Talwar et al. 2018; Kim et al. 2021; Li et al. 2022). Each factor

can play a role in determining what microbes inhabit the gut, and what roles they play in the gut lumen and in communicating with the host organism. Of the environmental associated factors specifically for fish, the surrounding water is known to influence the gut microbiome (Giatsis et al. 2015). Thus, the salinity (Dulski et al. 2020; Kim et al. 2021), pH (Sylvain et al. 2016), temperature (Steiner et al. 2022) and general composition of the water all contribute to the community membership of the gut microbiome. When one of these environmental factors are altered (Djokic 2024), it can also have an effect on the members of the microbial community of the gut, which in turn impacts the function of the consortium of the gut microflora (Kim et al. 2021).

Suspended sediment is naturally occurring in aquatic environments. However, due to anthropogenic changes through logging, dredging, and coastal development, in conjunction with rain events (Wenger et al. 2017; 2018), suspended sediment plumes in waterways are becoming a more common occurrence (Syvitski and Saito 2007). The sediment can stay suspended in the water for weeks (Reverdin et al. 2021). Depending on the time year, this disruption can impact larval development, as well as fish behavior such as foraging and predator and prey relationships (Utne-Palm 2002; Wenger et al. 2014; 2017). While some of the effects of suspended sediment on fish are known, whether suspended sediment impacts the gut, and specifically the gut microbiome of fishes exposed to it, is not well understood. In this study, I aim to investigate how exposure to suspended sediment alters the structure and composition of the gut microbiome in a freshwater fish: Zebrafish (*Danio rerio*). We examined the impacts of suspended sediment on the zebrafish enteric microbiome in a time series to examine 1) whether the length of time of exposure would gradually change the gut microbial diversity, and 2) whether the microbiome could recover to pre-exposure diversity, and how long that recovery would take.

Zebrafish were used as our model organism due to their established role in scientific research, particularly for studies on physiology and environmental impacts. The species is highly studied, providing access to a wealth of resources related to the gut microbiome (Rawls et al. 2004; Sharpton et al. 2021; Zhong et al. 2022). This made them an ideal subject for investigating

how an altered environment, such as one with suspended sediment, impacts the gut microbiome (Lescak and Milligan-Myhre 2017). This study will provide new insights into how suspended sediment impacts the gut microbiome of zebrafish and to explore the resilience and recovery of this microbial community. Furthermore, our experimental design will allow us to examine whether duration over a range of timed exposure from acute (days) to chronic (>1 week), in addition to timed recovery periods, matter to the gut microbiota.

METHODS

Experimental Design and Sample Processing

This experiment was designed to assess zebrafish gut microbiome community membership when the fish are exposed to suspended sediment across different durations and to determine the potential for subsequent recovery when the sediment-exposure ends. Adult zebrafish, sourced and maintained at the University of California, Irvine, were individually tagged with a subcutaneous visible implant elastomer (Northwest Marine Technology, Inc., Anacortes,WA) for unique identification (See Chapter Two). The study involved four exposure durations: one, three, seven, and 28 days. For each duration, fish were allocated to one of three treatment groups: control, suspended sediment exposed, or recovery. For each treatment condition, twelve fish were housed across three replicate one-liter tanks, resulting in four fish per tank. All tanks were part of two identical and adjacent aquarium systems that were on the same rack, in the same room, with the same source water reservoir, but with separate sumps. Both were re-circulating systems equipped with biological, activated carbon, and ultraviolet filtration, but lacking particulate filtration since a particle filter would trap the suspended sediment. Control groups were maintained in one system of sediment-free water for the specified duration, whereas suspended sediment exposed groups were housed in a system in which the water had a constant suspension of kaolin clay at a concentration of 45 mg/L, the concentration of which was monitored and maintained using a spectrophotometer (see Chapter 1). Other than the sediment, temperature and water quality parameters did not differ among the systems. The recovery group

fish were first exposed to the same sediment concentration for a set duration and then that tank was cleaned (fish were placed in a holding tank containing aquarium water for 10 minutes during cleaning) and transferred to the control system for an equal period of time before sampling (Fig. 1).

At the conclusion of each group's duration the fish were individually sacrificed in buffered water with tricaine methanesulfonate (MS-222, Argent Chemicals Laboratory, Inc., Redmond, WA, USA). The fish were then measured (total length, mm), and dissected on a chilled cutting board. Dissection surfaces and tools were cleaned in 100% ethanol and RNaseZap (Thermo Fisher), prior to dissecting each fish. The digestive tracts were removed, measured, weighed, and the distal portion (~25 mg) was fixed in DNA/RNA Shield at 4°C for 24 hours, then drained of excess liquid and immediately frozen in liquid nitrogen and stored at -80°C until DNA isolation.

DNA was isolated from samples using the ZymoBIOMICS DNA Mini Kit (Zymo Research) and using a TissueLyser II for the bead-beating step. Following DNA isolation, 16S ribosomal RNA gene amplicons (V3 and V4 regions) were prepared for sequencing on the Illumina MiSeq System according to the Illumina 16S Metagenomic Sequencing Library Preparation protocol. This involved a two-stage PCR amplification process. The first stage amplified the 16S rRNA gene regions using region-of-interest specific primers with overhang adapters. The PCR products were then purified using AMPure XP beads to remove unligated primers and primer dimers. In the second stage, dual indices and Illumina sequencing adapters were attached to the amplicons using the Nextera XT Index Kit through a limited-cycle PCR. A subsequent clean-up step with AMPure XP beads was performed to purify the final library. The prepared libraries were then quantified, normalized, and pooled before denaturing and loading onto the Illumina MiSeq system for paired-end sequencing.

The microbiome sequencing data was processed and analyzed using QIIME 2 (version 2024.10) on the University of California, Irvine's High-Performance Computing (HPC) cluster. Initially, raw paired-end fastq files were imported into QIIME 2 using a dedicated import function,

specifying a data type representing demultiplexed paired-end sequences with associated quality scores as the input type and a manifest-based format for paired-end fastq files with Phred33 quality scores as the input format. This step converted the raw sequencing reads into a QIIME 2 artifact. Following import, a quality check was performed by generating a summary visualization of the demultiplexed data for assessing read quality and guiding subsequent trimming parameters.

Adapter sequences and primers were then removed from the demultiplexed sequences using a paired-end trimming tool. Forward primer (CCTACGGGNGGCWGCAG) and reverse primer (GACTACHVGGGTATCTAATCC) sequences were specified, with an error rate of 0 and utilizing 12 cores for efficient processing. The trimmed sequences were saved as a QIIME 2 artifact.

Denoising and dereplication of the trimmed sequences were performed to generate Amplicon Sequence Variants (ASVs) using the DADA2 plugin. The resulting ASV table and representative sequences were then subjected to quality control. A visualization of denoising statistics was created by tabulating metadata for quality assessment. To ensure data robustness, features (ASVs) present in fewer than two samples were filtered out from the ASV table. Concurrently, corresponding sequences were filtered.

Taxonomic classification of the ASVs was carried out using a scikit-learn based feature classifier against the pre-trained SILVA 138 99% naive Bayes classifier, which was downloaded from the QIIME 2 data resources. The classification results were stored as a QIIME 2 artifact. Further filtering was applied to the ASV table and sequences to remove mitochondrial and chloroplast sequences, as these are typically considered contaminants in microbiome studies. This was achieved by filtering the taxonomic table to exclude these specific taxa, resulting in a filtered table and sequences.

A phylogenetic tree was constructed from the filtered representative sequences using a pipeline for aligning sequences and inferring a tree with MAFFT and FastTree. This involved

multiple sequence alignment with MAFFT, masking of highly variable regions, and subsequent tree inference with FastTree, yielding both an unrooted and a rooted phylogenetic tree.

Finally, diversity analyses were performed. Alpha rarefaction curves were generated using a tool for calculating alpha diversity with a maximum sampling depth of 16,000 reads per sample to assess sequencing depth and compare alpha diversity metrics across samples. Taxonomic bar plots were visualized using a tool for creating bar plots of taxonomic composition to illustrate the relative abundance of different taxa across samples. Beta diversity was calculated using the Bray-Curtis dissimilarity metric via a tool for computing beta diversity. Principal Coordinates Analysis (PCoA) was then performed on the Bray-Curtis distance matrix, and the results were visualized using an Emperor plot to explore community composition differences between samples based on metadata.

Statistical Analysis

All statistical analyses were performed in R using the vegan, dplyr, and ggplot2 packages. Alpha diversity was calculated using the Shannon diversity index. To test for significant differences, a series of one-way Analyses of Variance (ANOVAs) were conducted. First, the three treatments were compared within each of the four experimental durations. Second, the effect of experimental duration was assessed within each treatment group. Significant ANOVA results were followed by Tukey's Honest Significant Difference (HSD) post-hoc tests.

Beta diversity was assessed using a Bray-Curtis dissimilarity matrix and visualized with a three-dimensional Non-Metric Multidimensional Scaling (NMDS) plot. A Permutational Multivariate Analysis of Variance (PERMANOVA), conducted with the adonis2 function, was used to test for significant differences in community composition based on experimental duration. Finally, an indicator species analysis was performed using the multipatt function to identify microbial taxa significantly associated with each experimental duration. For all tests, a p-value of < 0.05 was considered statistically significant. Note that the seven day control fish cannot be used for this study because those fish were fasted prior to dissection, unlike the other treatments that

were fed before sampling. Thus, given that control fish should be unchanging over time, the other time points are good points of reference for the control condition. To visualize the distribution of unique and shared microbial species across the different experimental conditions, a Venn diagram was using the ggvenn R package. First, the OTU table was partitioned into three subsets based on the treatment category: control, suspended sediment, and recovery. For each subset, a list of all present species was compiled by identifying every OTU with an abundance value greater than zero in at least one sample within that group. These three distinct lists of species IDs were then supplied to the ggvenn() function, which calculated the intersections and differences between the lists and rendered a Venn diagram illustrating the number of species unique to each treatment and shared among them.

RESULTS

Alpha Diversity

The effect of treatment on Shannon diversity was first assessed within each experimental duration (Fig. 2). For the one day experiment, the overall model was statistically significant ($F_{2, 9}$ = 8.25, P = 0.009, figure 2A). Specifically, the recovery group had significantly lower diversity than both the control (P = 0.009) and sediment groups (P = 0.038). For the three day experiment, the model was also significant ($F_{2, 7}$ = 6.41, P = 0.026, figure 2B), with the control group showing significantly higher diversity than both recovery (P = 0.033) and sediment groups (P = 0.047). For the 28 day experiment, the effect of treatment was again significant ($F_{2, 8}$ = 5.51, P = 0.031, figure 2D), with the control group having significantly higher diversity than the sediment group (P = 0.041), but the recovery group was not different from either.

The effect of experimental duration on Shannon diversity was then assessed within each treatment group. No significant effect of duration was found for the control group ($F_{3, 7}$ = 0.307, P = 0.82 figure 2E) or the sediment group ($F_{3, 10}$ = 0.977, P = 0.44, figure 2F). In contrast, a highly significant effect of duration was observed for the recovery group ($F_{3, 12}$ = 11.9, P < 0.001, figure 2G), and the diversity in the one day experiment was significantly lower than in the 7-day (P =

0.002) and 28 day ($P < 0.001$) experiments.

Beta Diversity

Microbial community composition was visualized using a three-dimensional NMDS plot, which produced a good fit for the data (stress = 0.123). The plot showed considerable overlap among the sample groupings, with no distinct clustering by either treatment or experimental duration. A PERMANOVA based on Bray-Curtis dissimilarity was used to statistically test these observations. The overall model was not statistically significant ($F_{4, 37} = 1.546$, $R^2 = 0.14$, $P = 0.108$), but the 28-day sediment exposed group formed a distinct cluster in the NMDS (Fig. 3).

An indicator species analysis was performed to identify taxa significantly associated with the control, recovery, and sediment treatment groups. The analysis identified a total of 561 species and of that, 45 significant indicator species ($\alpha = 0.05$). For the control treatment there were 39 significant indicator species. The strongest association was observed for an uncultured member of the *NS11-12 marine group* (stat = 0.608, $P = 0.0003$), followed by an unclassified species from the *Gammaproteobacteria* class (stat = 0.531, $P = 0.0004$). Other highly significant indicators for the control condition included uncultured members of the *Flavobacteriaceae* family (stat = 0.509, $P < 0.001$).

In the sediment group, three taxa were identified as significant indicators. An uncultured member of the Rhodobacteraceae family showed the strongest association (stat = 0.541, p = 0.0011). A species from the genus Rhizobacter (stat = 0.314, p = 0.0468) and an uncultured member of the Cyclobacteriaceae family (stat = 0.376, p = 0.0490) were also significantly associated with the sediment samples.

The recovery group was similarly characterized by three indicator species. The most significant association was for an uncultured member of the Gemmataceae family (stat = 0.401, p = 0.0304). A taxon within the Enterobacterales order (stat = 0.350, p = 0.0327) and a member of the Rhodobacteraceae family (stat = 0.341, p = 0.0393) were also identified as significant indicators for this group. An unclassified species of *Gammaproteobacteria* (stat = 0.452, *P*=

0.0007) was also associated with the recovery group.

Furthermore the control group had 25 unique species that particular group. The sediment group had 7 unique species, while the recovery group had 102 unique species. There 211 species present in all groups, 130 species shared between the control and recovery treatment, 26 species shared between the control and sediment and 51 species shared between the sediment and recovery treatment (Fig. 5).

DISCUSSION

In this study, we tested what happens to the gut microbiome of zebrafish when exposed to suspended sediment across four different durations of time from acute to chronic. Additionally, we tested a potential recovery of a gut microbiome that was disrupted due to suspended sediment exposure, and how long such a recovery could take. We hypothesized that the overall community membership would change when exposed to suspended sediment and that there would be a gradual recovery phase, where community membership could return to resembling what was found in the control group if given enough time.

For the one day and three day experiment we see a decrease in alpha diversity in the recovery treatment, however in the seven day and 28 day experiment we see where there's no significant difference in alpha diversity between the control and recovery treatments. Based on this we see that it took seven days for previously sediment exposed fish to regain community membership similar to the control treatment. Microbiomes generally don’t change immediately (Xia et al. 2014; Wong et al. 2015; Stagaman et al. 2020). For instance, after exposure to a broad spectrum antibiotic, it took two months for zebrafish gut microbial diversity and function to be restored (Almeida et al. 2021), yet zebrafish (Wong et al. 2015) and other fish species can show shifts in microbiomes in a matter of days in response to other environmental changes (Clements et al. 2014; Xia et al. 2014).

In terms of alpha diversity, the recovery group showed lower alpha diversity after one day than the control or sediment groups, which did not differ at this time point (Fig. 2). This implies

that quick environmental shifts disrupt alpha diversity in the short term. As time progressed, the alpha diversity of the sediment-exposed fish dropped and stayed lower than the control for the remainder of the experiment, whereas the recovery group's alpha diversity recovered by day seven and 28 (Fig. 2), showing that after the initial perturbation, the diversity recovered.

The suspended sediment and recovery group were not different at day seven. Perhaps there was an adjustment in microbial diversity. As we increased the duration of the 28 day experiment, there was no significant difference in the control and recovery, yet the sediment-exposed fish maintained a lower alpha diversity than either group. The control group represents a stable and well-defined baseline community. This is evidenced by the high number of significant indicator species (39 different ASVs), which serve as reliable biomarkers for this undisturbed state. Furthermore, this group contains 25 unique species (Table 1) that are not found in the other two treatments (Fig. 5). These unique taxa, including members of the genera *Lactococcus* and *Brevibacillus*, represent the sensitive portion of the baseline community that was lost or suppressed following the sediment exposure in the other two groups. Specifically, the Rhizobiales order, first found in the roots of plants and known to fix atmospheric nitrogen, are a part of the community membership in the control group (Garrido-Oter et al. 2018). This order of bacteria is also known to be a dominant player in biofilm communities, and documented in to be found in omnivorous fish (Pang and Liu 2007; Kononova et al. 2019; Kormas et al. 2023). Many studies have documented this bacterial order in zebrafish guts across different laboratory facilities (Stephens et al. 2016; Sharpton et al. 2021). The fact that this group disappeared from the sediment and recovery fish (regardless of duration) shows that this group may be sensitive to perturbations and their role in the zebrafish fish gut should be explored in more detail. While the control group has unique species not found in the sediment and recovery group, across studies it is consistent with having common microbes known as a core microbiome. Members of the Pseudomonodota, Fusobacteriota, Bacillota, Bacteroidota, and Actinobacteriota were all present (Rawls et al. 2004; Roeselers et al. 2011; Sharpton et al. 2021).

A unique species to the suspended sediment group was an uncultured member of the family of Rhodobacteraceae that are prominent in marine and aquatic habitats and play roles in biogeochemical cycles, especially those involving sulfur and carbon (Pujalte et al. 2014). Furthermore Rhodobacteraceae are dominant players in biofloc microbiota and most members of this family encode full pathways for central carbohydrate metabolism, including glycolysis, the TCA cycle, and the pentose phosphate pathway, confirming their heterotrophic nature and contribution to organic matter degradation. Additionally, members of this family are primarily involved in the reduction of nitrate to ammonium and denitrification (Rajeev and Cho 2024). They are known from omnivorous fish guts (Rankins et al. 2023). Another family, Cyclobacteriaceae, found only in the sediment group, is known for their ability to degrade complex organic matter (Pinnaka and Tanuku 2014).

The recovery group displayed the classic pattern of a community in a state of ecological transition. It had the lowest number of reliable indicator species (3), suggesting a lack of community-wide stability. However, it contained the highest number of unique species (93) (Fig. 5). This combination indicates a highly variable and dynamic environment being colonized by many different species that have not yet formed a consistent, stable community membership.

Of the things that influence the community composition of the gut microbiome are generally broken down into three categories of diet, environment and the host, we explored time dependent environmental alterations, which would certainly fall into the environmental category (Talwar et al. 2018; Kim et al. 2021; Small et al. 2023). Although we have gained insight from this experiment, it needs to be replicated and scrutinized. One of the main variables that influence captive fish microbiomes is the aquarium itself (Small et al. 2023) and there is evidence of a core microbiome within zebrafish regardless of different housing (Breen et al. 2019; Sharpton et al. 2021). However, beyond the sediment treatment itself, our results could simply reflect two different aquarium systems (one with sediment and one without), meaning that, despite controlling for “tank” in our design, it is somewhat pseudoreplicated (Hurlbert 1984). However, the close

proximity (literally centimeters apart in the same room), and similar water quality (other than the sediment) argues that we did what we could to make them as identical as possible other than suspended sediment, which can still allow for microbial variation among fish in the different systems (Small et al. 2023). Another limitation to this experiment was that water samples (Wong et al. 2015; Djokic 2024) from each system were not taken for microbial analyses, and thus a microbial comparison between the systems cannot be taken into account. Fish gut microbiomes commonly don't resemble the water in which the fish are housed (Sullam et al. 2012; Wong et al. 2015; Li et al. 2022; Djokic 2024) due to the selective environment of the fish gut, but such samples would indicate how different the systems were from each other. Certainly, physical variables, such as turbidity, impact the diversity and function of riverine microbiomes (Borton et al. 2025).

In conclusion, anthropogenic pressures on the aquatic ecosystems are ever increasing. Dredging, logging, and overall infrastructure development, are altering the way ecosystems function, particularly with regards to suspended sediment in waterways (Wenger et al. 2014; 2017; 2018; Borton et al. 2025). In order to implement proper management strategies, it is important to understand how various perturbations impact the organisms in affected systems. At the very least, this study, in conjunction with the other chapters, suggests that suspended sediment affects the zebrafish intestinal microbiome, which can have cascading effects on fish physiology. Our finding that a seven day recovery time to return alpha-diversity to pre-exposure values is encouraging, but the fact that some community members never returned (i.e., members of Rhizobiales) provides insight for potential recovery plans following storm or dredging events. More detail is needed on those taxa most impacted and how they affect the fish host, but perhaps a probiotic could be added to waterways following sediment inputs to hasten their return after the perturbation.

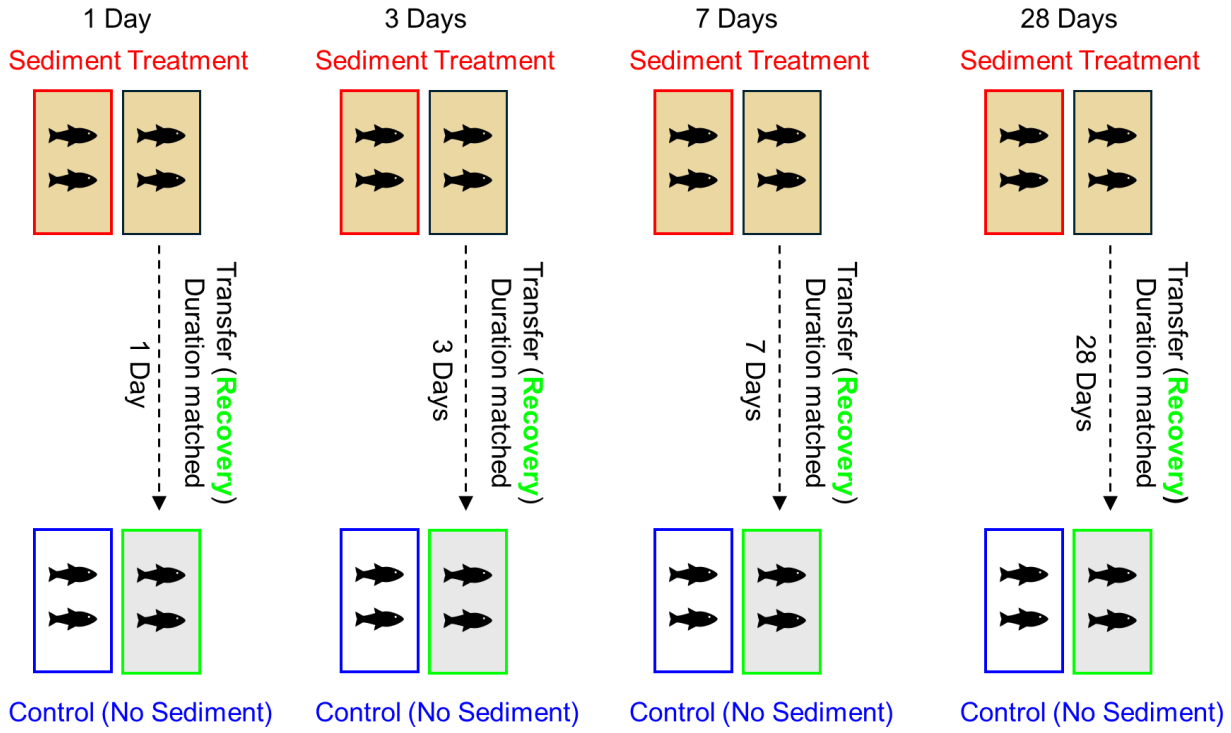

Figure 1. Experimental design of the treatment (control, sediment and recovery), through the various durations of time.

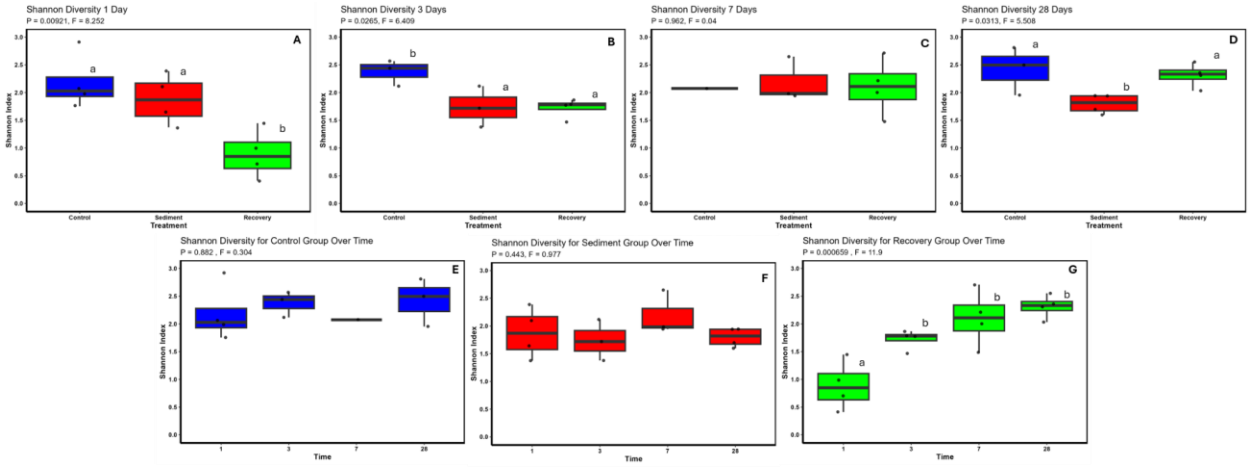

Figure 2. Shannon diversity index of the zebrafish gut microbiome across different experimental conditions. The panels are organized into two sections. Panels (A) through (D) show the effect of treatment on Shannon diversity within each experimental duration: (A) one day, (B) three day, (C) seven day, and (D) 28 day. Panels (E) through (G) show the effect of experimental duration on Shannon diversity within each treatment group: (E) control, (F) suspended sediment, and (G) recovery. Within each graph, values were compared with ANOVA followed by Tukey's HSD, and boxes not sharing a letter are statistically significantly different ($P<0.05$).

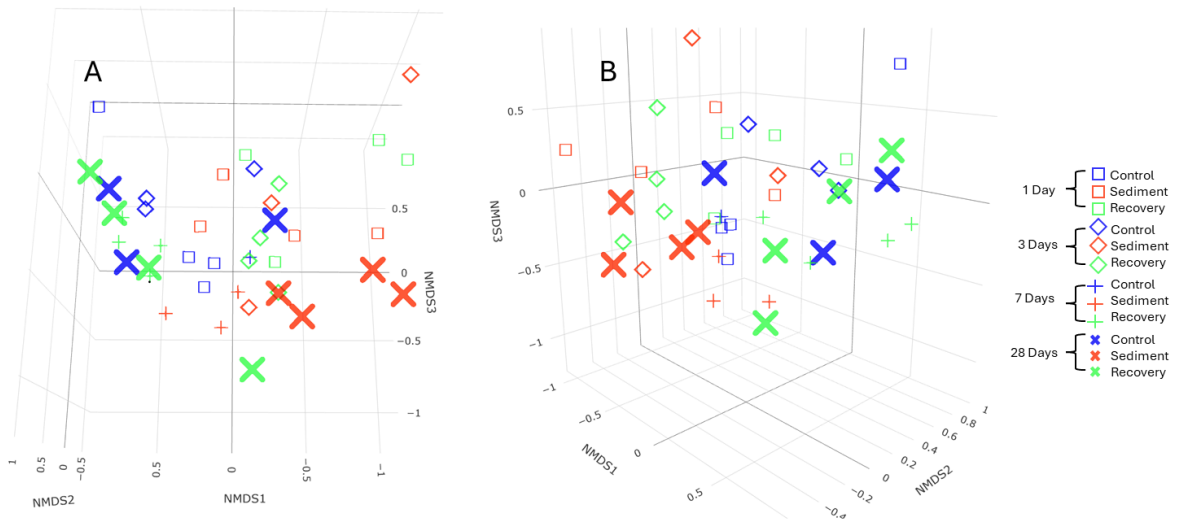

Figure 3. Three-dimensional Non-Metric Multidimensional Scaling (NMDS) plot visualizes the beta diversity, among all samples. The points are colored according to the treatment group (control, suspended sediment, or recovery) and shaped by the experimental duration (one, three, seven, or 28 days). The two panels are the same graph but rotated to better see the divide along NMDS1 and NMDS3 (especially in Panel A). Longer duration sediment exposure is lower on NMDS1 and NMDS2, whereas longer duration control or recovery is higher on these axes (Panel A).

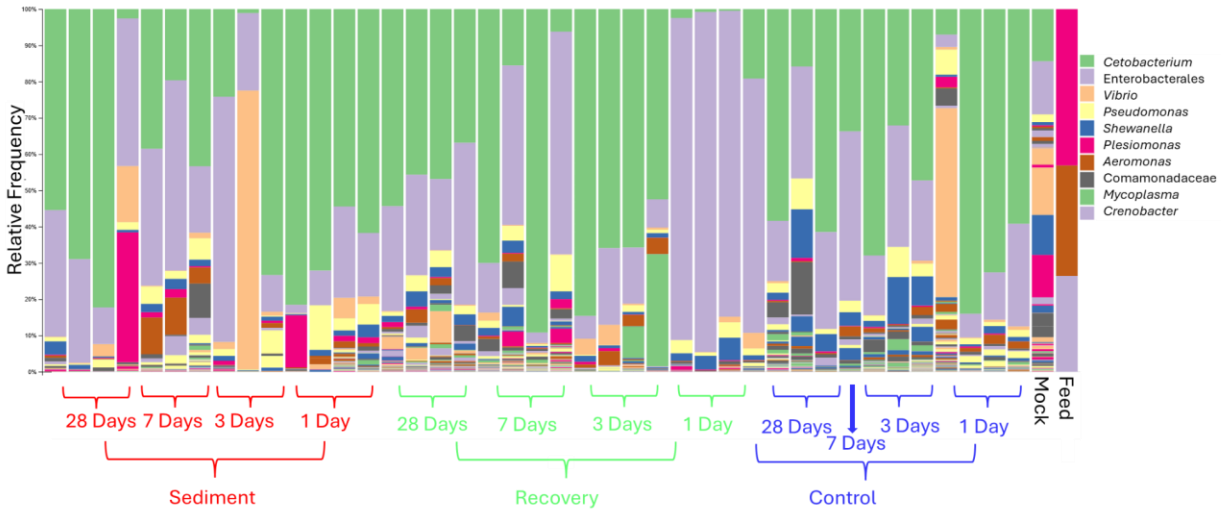

Figure 4. Relative abundance of different bacterial families within a microbial community across various samples. Each vertical bar represents a single sample. The height of each colored segment within a bar corresponds to the relative abundance of a specific bacterial family. The legend shows the top ten most abundant taxa to the lowest classification. (For full ASV breakdown http://german.bio.uci.edu/images/PDF/Zebrafish_ASV_all.csv)

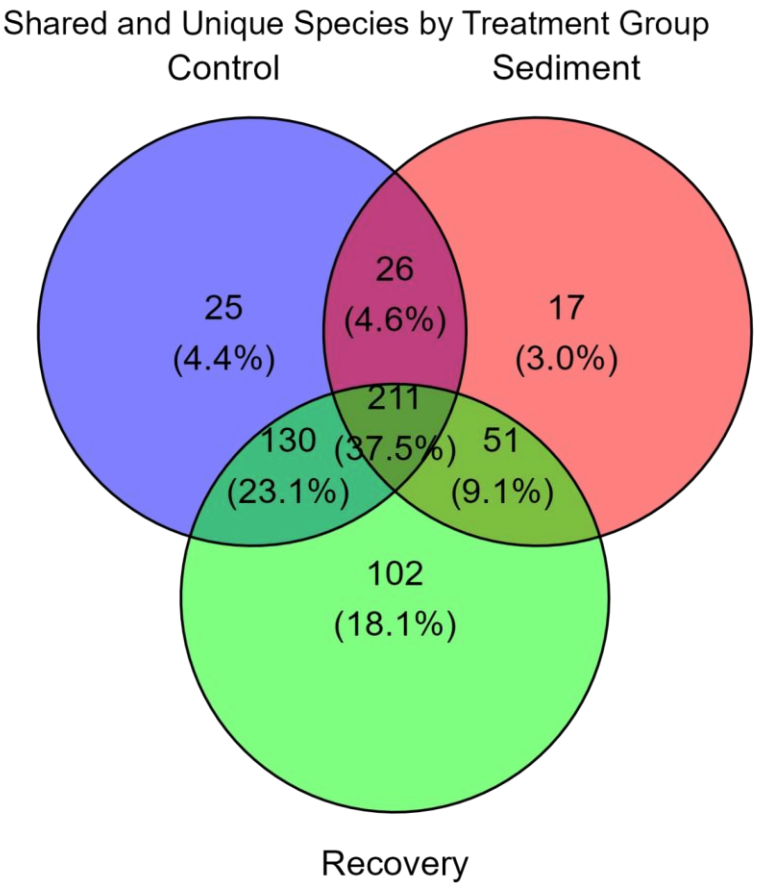

Figure 5. Venn Diagram of shared and unique species by treatment group.

Table 1. The unique bacterial taxa represented in the guts of zebrafish from each treatment in the experiment. Each taxonomic level is listed once, although there can be multiple ASVs from that taxonomic level, with the number of ASVs indicated in parentheses.

Control
Pseudomonodota
Rickettsiaceae
Novispirillum
Salinisphaera
Comamonadaceae
AAP99
Actinobacteriota
PeM15
IMCC26256
Dependentiae
Babeliales
Vermiphilaceae
Planctomycetota

Gemmataceae
uncultured
Bacillota
Lactobacillus
Suspended Sediment
Pseudomonodota
Procabacter
Enterobacteriaceae
Fusobacteriota
Cetobacterium
Recovery
Pseudomonodota
Acinetobacter (3)
Candidatus
Berkiella (2)
Pseudomonas (2)
Sphingomonas
Photobacterium
Ideonella
Hyphomicrobium
Gammaproteobacteria
Actinobacteriota
Turicella
Rothia
Corynebacterium
Brevibacterium
Microbacteriaceae
Dietzia
Nocardioides
Bacteroidota
AKYH767
Emticicia
Flavobacterium
Edaphobaculum
Spirosoma
Bacillota
Latilactobacillus
Lactobacillus
Enterococcus
Aerococcus
Planctomycetota

Isosphaeraceae
OM190
Verrucomicrobiota
DEV008
Chthoniobacter
Acidobacteriota
Subgroup 17
Bdellovibrionota
Bdellovibrio
Desulfobacterota
Desulfovibrionaceae
Patescibacteria
Candidatus Peribacteria
WPS-2
WPS-2

CONCLUSION

Anthropogenic activities are altering the natural world, and thus, it is important to investigate how our activities impact organisms like fish, which will inevitably affect humans. This dissertation was an exploration of what happens when zebrafish are exposed to suspended sediment. In the initial investigation of chapter one, I explored what happens to zebrafish when exposed to a chronic (28 days) and known sublethal level of suspended sediment.

- Chronic (28-day) exposure to suspended sediment acts as a significant gastrointestinal perturbation rather than just a diluent of food.
- Sediment exposed fish exhibited significantly reduced final body lengths and shorter relative gut lengths, supporting the "perturbation hypothesis" that fish consumed less food to avoid irritation.
- A significant reduction in alanyl-aminopeptidase activity was observed, indicating a compromised ability to digest and absorb proteins.
- A qualitative increase in gut goblet cells was noted, signaling a potential protective response.
- The absence of elevated whole-body cortisol or gill heat shock proteins suggested that suspended sediment was not a chronic stressor, however this is explored more in chapter two.

In chapter two, building on the preliminary chapter, I tested what the physiological effects of suspended sediment exposure on acute (one, three and seven days) and chronic (28 days) time scales with an equally matched time period to recover from the exposure in sediment-free water.

- Three day time duration gill *hsp70* expression increased significantly, with a non significance in the following treatments, suggesting a stress response then acclimation.

- Tracking gut length and digestive enzyme activity over time and during recovery, highlighted the digestive system's plasticity while also revealing the potential for lasting impairment in digestive function, especially for protein (trypsin) and lipid (lipase) digestion.
- The temporal data overall addressed questions from the previous chapter demonstrating that the 28 day endpoint is the result of a dynamic physiological process showing that suspended sediment may be an acute stressor for the gill, but a chronic perturbation for the gut.

In my third chapter, I investigated the effects of suspended within the gut microbiome through the time series and recovery experiment explained in chapter two.

- Alpha diversity decreased in sediment exposed fish and remained low. In contrast, the recovery group's diversity returned to control levels within seven days, establishing a key timeframe for resilience of general alpha diversity
- The recovery group showed a classic pattern of ecological transition, with low stability (few indicator species) but high variability (93 unique species) as new taxa colonized the gut environment.
- While alpha diversity increased by day 28, taxa in the order *Rhizobiales* never returned, showing that suspended sediment can indeed have lasting impacts on the gut microbiome of fish. These kinds of changes need to be taken into account when considering how fish recover from suspended sediment exposure.

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