Ibuprofen As A Water Pollutant On The Defensive Behaviour And Microbiome Of Grass Shrimp *Palaemonetes* Spp.

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ABSTRACT

Over-the-counter use of painkillers dates to the 1900s, and has become increasingly normalized in modern society. The first-pass effect coupled with insufficient sewage treatment mechanisms have led to significant levels of pharmaceutical pollution in the environment. Scientists are only recently delving into the implications of chronic use on the human gut microbiome, and similar ramifications may hold for marine life that ingest pharmaceutical waste. As one of the first research efforts to understand the effects of non-steroidal anti-inflammatory drugs (NSAIDs) on the gut microbiome of common marine species, this study points to potential microbiome alterations that may be correlated to behavioural complications from ibuprofen pollution.

INTRODUCTION

With increasing environmental conservation efforts, the body of research surrounding the impact of personal care and pharmaceutical products (PCPPs) pollution in natural waters on humans and other organisms has grown significantly in recent years. 19% of U.S adults are estimated to be chronic users of NSAIDs(1), of which ibuprofen is one. While this behaviour has long been linked to stomach ulcers, intestinal bleeding and perforation(2), recent research has shown effects on human gut microbiome profiles, specifically an increased abundance of *Pseudomonadaceae*, *Puniceicoccaceae* families(3). Altered microbial expression has yet to be definitively linked, however, to gastrointestinal effects in humans.

Seawater harbours up to 3 µg/L of ibuprofen(4), making it one of top eight PCPP contaminants (5). This study aims to understand the effect of different ibuprofen concentrations on *Palaemonetes* spp., a common grass shrimp.

Current literature on the effects of PCPPs on marine life focuses on direct consequences for the organism itself, such as decreased motor skills(6), metabolic disorders(7), endocrine disruption, and oxidative stress(8), with limited investigation of effects to the host's microbiome. Thus, my hypothesis is two-fold: firstly, the shrimp will exhibit lethargic and delayed defensive mechanisms with increasing ibuprofen concentration. Secondly, taxonomic abundance of *Pseudomonadaceae*, *Puniceicoccaceae* families in the shrimps' microbiome will increase with increasing ibuprofen concentrations.

METHODOLOGY

Sample Collection

Twenty-four shrimp were collected off the dock at the Marine Resources Center at the Marine Biological Laboratory (MBL), in Woods Hole, Massachusetts. Six shrimp each were put into four three-liter ambient seawater tanks dosed with ibuprofen at final concentrations of 0 (Control-IB), 3 (Low-IB), 21 (Medium-IB), and 80 (High-IB) mg/L respectively. Ibuprofen was added using powdered 200 mg dosage Advil tablets.

To sample the gut microbiome, I extracted the digestive tract through dissection at the time of death or after five days, whichever occurred earlier. Three samples were collected per treatment, and stored at -80°C.

Behavioural Analysis

In response to perceived threats, shrimp flick their tails and jump a horizontal distance (9). Throughout the experiment, the shrimp were tested regularly on their defence mechanisms by inserting a glass rod into the water and moving it closer to individual shrimp. Two metrics were recorded: perceived threat – the distance between the rod and the shrimp right before it jumps; the defence distance – defined as the distance jumped following the disturbance.

DNA isolation, 16S rRNA amplification, Illumina MiSeq sequencing

DNA was extracted following manufacturer protocol for the QIAGEN DNeasy PowerSoil kit (10), except the entire shrimp gut was used in place of 0.25 g of

sample to avoid contamination. The V4 region of 16S rRNA gene was amplified with primers 515F and 816R, following 16S Illumina Amplicon protocol in the Earth Microbiome Project (11). DNA concentration was confirmed with Nanodrop (12), PicoGreen assay (13) and agarose gel electrophoresis (14). PCR products were sequenced on the Illumina MiSeq platform in the Bay Paul Center at the MBL (15).

Sequencing Data and Statistical Analysis

Sequence data was demultiplexed using the “demux emp-paired” command (16). One Low-IB sample was lost due to low yield. The DADA2 plugin in QIIME2-2018.2 (17) was used to group related sequences into exact sequence variants (ESVs). All sequences with Phred scores below 25 were excluded, and taxonomy was assigned using the Greengenes database v13.8 (18). Sequences unidentifiable beyond kingdom level were discarded. Rarefaction curves using the Shannon H index (19) were plotted to ensure the filtered data accurately represented samples’ microbial diversity.

I used UniFrac distances (20) to examine inter-sample taxonomic differences, alongside principal coordinate analysis (PCoA) (21) and permutational multivariate analysis of variance (PERMANOVA) (22). Power calculations were conducted using HMP R package (23) and G*Power ANOVA (24).

RESULTS

Sequencing Analysis

Alpha and Beta Diversity

Shannon index analysis across the concentration gradient yielded no significant differences between any treatment groups, with an average index of 6.68 at insignificant p-value 0.082. Between dead and live samples however, there was a significant H-index of 7.5 and p-value 0.006.

The weighted Unifrac metric (Figure 1) showed more meaningful clustering compared to the unweighted counterpart. Statistically significant clustering was not observed between treatments, but was observed between live and dead samples. Thus, both dead and live shrimp microbiome profiles show intra-group similarity in

phylogenetic distance and relative taxonomic abundance, but live profiles are more heterogenous.

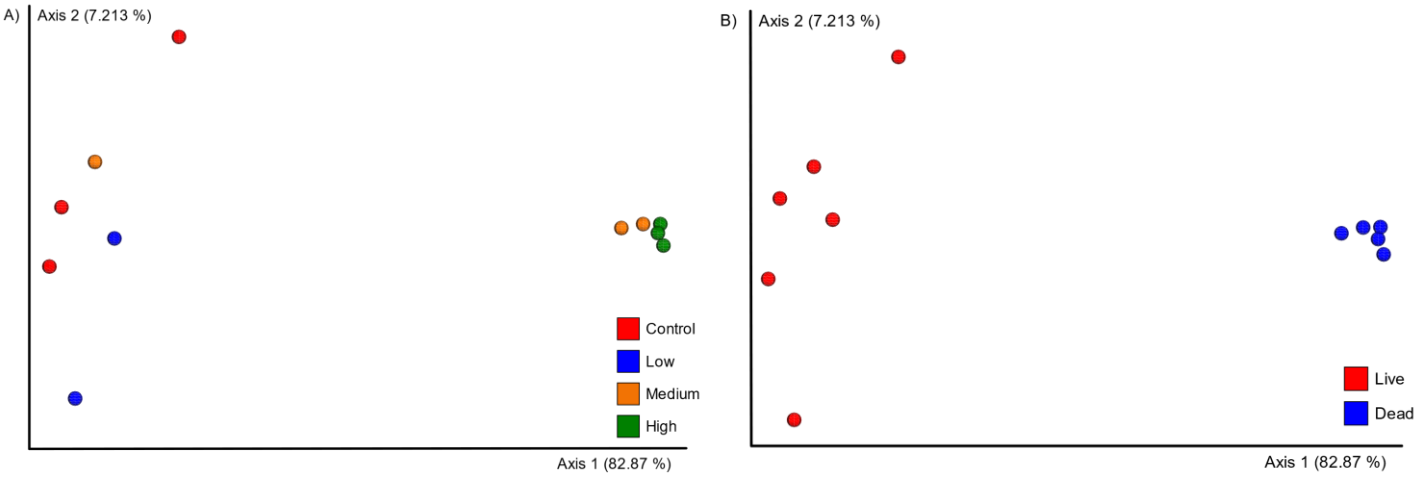

Fig. 1: A) Plot of principal coordinate analysis (PCoA) from weighted Unifrac showing insignificant clustering of the gut microbiome profile difference between treatments, with pairwise PERMANOVA p-values above 0.05 threshold. B) Significant gut microbiome profile difference between live and dead samples observed, with significant p-value of 0.001.

Differential taxonomic abundance

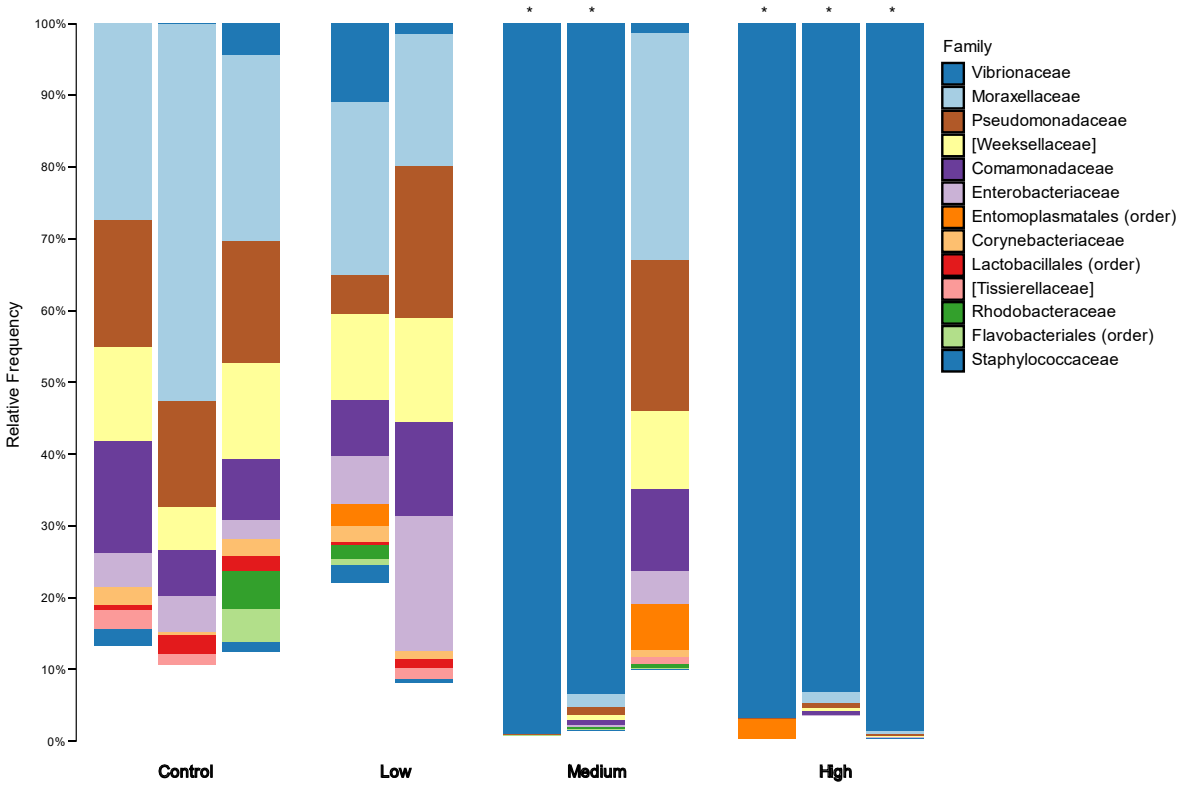

Fig.2: Taxonomic composition of samples by treatment. Asterisks denote samples that were dead upon collection. Only top 13 most abundant taxa are shown.

Dead shrimp gut microbiota (Figure 2) has lower average taxonomic diversity, and is overwhelmingly populated by *Vibrionaceae* (96.2%). Average relative abundances are denoted in parentheses.

The gut microbiome of live shrimp is more diverse, with significant microbial abundances of *Moraxellaceae* (30.1%), *Pseudomonadaceae* (16.1%), *Weeksellaceae* (11.72%) and *Comamonadaceae* (10.5%). *Vibrionaceae* (3.02%) was the sixth most abundant taxa, and notably contain a different, though unidentified, strain compared to dead counterparts.

ANCOM analysis (25) of the live shrimp (data not shown) did not identify any ESVs that differed significantly between treatments.

Behavioural Analysis

All shrimp in High-IB were dead by day 2, and 8 molts were deposited. On day 3, 5 shrimp in Medium-IB had died, and 4 molts were deposited. 1 molt in Low-IB and 0 molts in Control-IB were deposited by day 6.

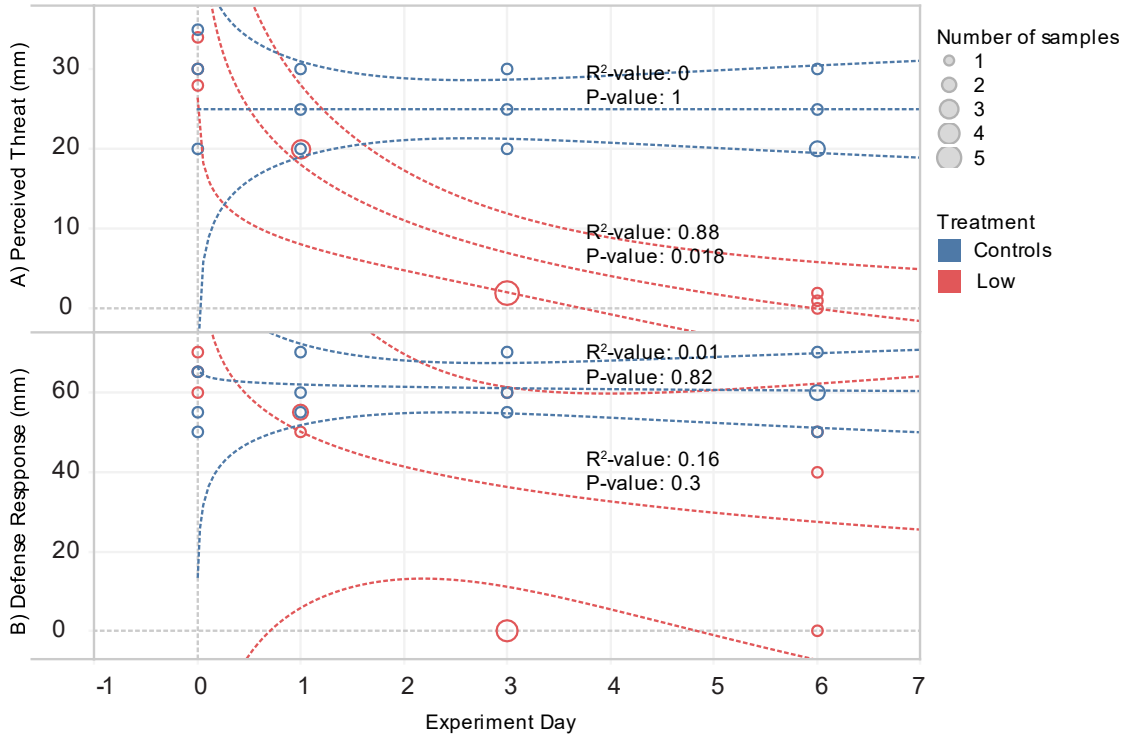

Fig. 3: A) Perceived threat distances measured for Control-IB and Low-IB treatments on day 0, 1, 3, 6 of the experiment. Each record corresponds to one shrimp. Low-IB showed statistically significant decreasing relationship with time at 0.05 p-value threshold. **B)** Defence distances measured under the same conditions as (A).

Logistic regression of behavioral data (Figure 3) showed visually negative correlations between ibuprofen concentration and defence capabilities, though only the trend for perceived threat was statistically significant.

DISCUSSION

Along the concentration gradient for live samples, I was unable to identify statistically significant differences in taxonomic abundances and diversity. However, the microbiome profiles of live and dead samples did differ significantly, especially in the abundance of *Vibrionaceae.* While a form of predominant marine bacteria, research has also shown that many strains are naturally virulent, and marine hosts have evolved mechanisms to restrict pathogenesis (26). It is therefore possible that higher fatality relative to concentration is linked to *Vibrionaceae* spp. by compromising host immunity, aiding vibrio pathogenesis – by either colonization of external strains or once commensal strains. It is also possible that the predominant strain observed in the dead samples is commonly found in decaying grass shrimp gut microbiome.

However, significant physical effects were observed. High mortality rates in High-IB and Medium-IB show that ibuprofen is lethal for grass shrimp at high concentrations. Ibuprofen ingestion can compromise host survival in the face of predators also, shown by significantly shorter perceived threat distances and visibly lowered defence distances measured by Low-IB compared to Control-IB. Lastly, the alarming count of shrimp molts in High-IB and Medium-IB suggests that ibuprofen can indirectly alter the circadian rhythm of the molting cycle. Given that the regular molting cycle ranges from fourteen to twenty-one days (27), the newly developed shells may be lacking in protective capabilities and may compromise survival. Further research is needed to determine if weakened external shells are correlated to or accelerate shrimp death.

I was underpowered to conclude significant differences in alpha and beta diversity. Power analysis using G*Power ANOVA on the Shannon H index across the concentration gradient for live samples only yielded 0.11 power, and the minimum sample size for 0.8 power given my effect size is 36. Using Dirichlet-Multinomial

parameter test on the beta diversity across the concentration gradient yielded 0.50 power. Thus, these results are best taken as a starting point for further research.

Aside from sample size, a few alterations should be made to the experimental design in further research. Firstly, the duration of the experiment should be increased. Secondly, the experiment should be conducted with seawater diluted by pure ibuprofen salt concentrations as well as powdered Advil solutions. While ibuprofen is the active ingredient in Advil tablets, I cannot exclude the possibility that some trace ingredient in the tablets, undigested by the human body, is the true culprit for host compromise and fatality in this experiment. Should this be true, this could have implications for pharmaceutical companies as they revise peripheral ingredients in pills and for waste management systems filtering for pharmaceutical contaminants.

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