Spring 2023

The effects of diatom-specific polyunsaturated aldehydes on larval zebrafish (Danio rerio)

Rachel Raymer
Western Washington University, r.rachel.r@gmail.com

Follow this and additional works at: https://cedar.wwu.edu/wwuet

Part of the Biology Commons

Recommended Citation

This Masters Thesis is brought to you for free and open access by the WWU Graduate and Undergraduate Scholarship at Western CEDAR. It has been accepted for inclusion in WWU Graduate School Collection by an authorized administrator of Western CEDAR. For more information, please contact westerncedar@wwu.edu.
The effects of diatom-specific polyunsaturated aldehydes on larval zebrafish (*Danio rerio*)

By

Rachel Raymer

Accepted in Partial Completion
of the Requirements for the Degree
Master of Science

ADVISORY COMMITTEE

Dr. Brady Olson, Chair

Dr. James Cooper

Dr. Karin Lemkau

GRADUATE SCHOOL

David L. Patrick, Dean
**Master’s Thesis**

In presenting this thesis in partial fulfillment of the requirements for a master’s degree at Western Washington University, I grant to Western Washington University the non-exclusive royalty-free right to archive, reproduce, distribute, and display the thesis in any and all forms, including electronic format, via any digital library mechanisms maintained by WWU.

I represent and warrant this is my original work, and does not infringe or violate any rights of others. I warrant that I have obtained written permissions from the owner of any third party copyrighted material included in these files.

I acknowledge that I retain ownership rights to the copyright of this work, including but not limited to the right to use all or part of this work in future works, such as articles or books.

Library users are granted permission for individual, research and non-commercial reproduction of this work for educational purposes only. Any further digital posting of this document requires specific permission from the author.

Any copying or publication of this thesis for commercial purposes, or for financial gain, is not allowed without my written permission.

Rachel Raymer

4/30/2023
The effects of diatom-specific polyunsaturated aldehydes on larval zebrafish (*Danio rerio*)

A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

by
Rachel E. Raymer
May 2023
ABSTRACT

Diatoms are ubiquitous in marine planktonic and benthic environments and are common in diets for many lower-trophic organisms. Certain species of diatoms produce polyunsaturated aldehydes (PUAs) that can exist in particulate and dissolved forms. Diatom PUAs are known for negatively affecting the fecundity of their primary consumers, including invertebrate grazers like copepods and echinoderms. However, little is known about the effects of diatom PUAs on vertebrates that may be exposed to dissolved or ingested PUAs due to overlapping distribution with diatom populations. The purpose of this study was to test whether dissolved diatom PUAs affect the early life stages of a model fish, *Danio rerio* (zebrafish). To test this, zebrafish embryos and larvae were exposed to proportionally increasing mixtures of the dissolved diatom PUAs 2E,4E-decadienal, 2E,4E-octadienal and 2E,4E-heptadienal. Under PUA exposure three metrics of fitness were assessed: embryo heart rate, larval size at hatch, and pre-feeding rate of mortality. In the embryo heart rate experiment, embryos exposed at 24 hours post fertilization (hpf) experienced decreased average heart rate after 2 days of PUA exposure. Embryos 24 hpf exposed to PUA mixtures for 6 days showed a reduction in size in comparison to embryos from controls. Embryos exposed to PUAs from 2 hpf until death showed lower survivorship compared to larvae in controls. The results of this study suggest that larval fish that are sympatric with PUA producing diatoms during their embryonic and larval stages may be susceptible to detrimental effects from PUA exposure.
Acknowledgements

I would like to acknowledge first and foremost my advisor, Dr. Brady Olson, for sticking with me throughout this experience and not letting me give up. I would also like to thank Dr. James Cooper for the endless stream of zebrafish embryos on which to base this research. And last but not least, Dr. Karin Lemkau, for assisting with the chemistry side of this project.

Thank you also to my undergraduate assistant (now a Bachelor of Science!) Soraya Jessa for helping me record and analyze 100+ fish heart videos, and for being willing to jump in and help on any other projects I had going on.

I also can’t forget my fellow Olson lab mate (and chemist) Jeremy Johnson, who rescued me from some last-minute concentration recalculations when it seemed like there was no hope.

My thanks, also, to Juan Pablo Carreón Hidalgo at Universidad Autónoma Metropolitana, Iztapalapa. Even though I was a complete stranger hundreds of miles away, he was happy to lend me his R expertise multiple times to help me make the perfect visuals of my nested data.

And, of course, to my friends and family.

Science is conducted by communities, and this thesis would not exist without the support of these wonderful people. Thank you!
# Table of Contents

Abstract ........................................................................................................................................ iv

Acknowledgements ....................................................................................................................... v

List of Figures .................................................................................................................................. vii

List of Tables ................................................................................................................................. viii

Introduction ....................................................................................................................................... 1

Methods .......................................................................................................................................... 6

Results ............................................................................................................................................. 13

Discussion ......................................................................................................................................... 18

Figures and Tables .......................................................................................................................... 24

Works Cited ....................................................................................................................................... 38
List of Figures

Figure 1: Pathways for embryonic and larval forage fish interactions with PUAs..........................24

Figure 2: Developmental experiment designs for PUA effects on zebrafish larvae......................26

Figure 3: Diagram of the morphometric dimensions measured for larval size..............................26

Figure 4: Data from heart rate experiment ..................................................................................28

Figure 5: Data from larval standard-length measurements..........................................................29

Figure 6: Data from deepest vertical point measurements............................................................31

Figure 7: Data from myotome height at the anus measurements..................................................33

Figure 8: PCA plot of the variation in size of zebrafish larvae based on PC1 and PC2..............36

Figure 9: Kaplan-Meier survival probability curves.................................................................37
List of Tables

Table 1: PUA mixture concentrations for experiments .................................................................25
Table 2: Linear mixed model results for heart rate ........................................................................27
Table 3: Linear mixed model results for standard length ..............................................................30
Table 4: Linear mixed model results for deepest vertical distance ..............................................32
Table 5: Linear mixed model results for myotome height at the anus ........................................34
Table 6: PERMANOVA pairwise comparison results .................................................................35
Table 7: Standard deviation, proportion of variance, and cumulative proportion of principal components analysis ........................................................................................................35
Table 8: Kaplan-Meier analysis pairwise comparisons ..............................................................37
INTRODUCTION

Diatoms are a lineage of microscopic algal phytoplankton that are an important group in marine ecosystems because of their basal trophic position as primary producers and their prominent role in global biogeochemical cycles. Diatoms produce more oxygen than all of the world’s rainforests combined, support global fisheries, and contribute to the biological carbon pump by sequestering atmospheric carbon dioxide in their bodies, which sink to depth upon cell death (Field et al., 1998; Smetacek, 1999; Tréguer et al., 2017). They can be especially abundant in coastal waters due to the increased presence of nutrients from terrestrial environments and from seasonal upwelling (Malviya et al., 2016). This high biomass of diatoms in coastal environments means strong competition pressure for resources within diatom communities and from other primary producer functional groups (Zhou et al., 2017).

To gain a competitive advantage for resources, some diatoms produce and emit bioactive chemicals that can have effects across trophic levels (Pohnert, 2005; Franzé et al., 2018). These chemicals can lead to reduced herbivore grazing and decreased growth of co-occurring phytoplankton populations (Ribalet et al., 2007a; Romano et al., 1999). Oxylipins, a compound within this array of diatom-produced chemicals, are a product of the lipoxidation of polyunsaturated fatty acids (PUFAs). Oxylipins have displayed high bioactivity in plants, algae, and animals (Barbosa et al., 2016; Prost et al., 2005; Tourdot et al., 2014). Some diatoms, upon damage to the diatom cell wall, intracellularly produce oxylipins called polyunsaturated aldehydes (PUAs), biomolecules composed of a chain lipid with an attached oxygen atom (Caldwell et al., 2009; Fontana et al., 2007). The length of the lipid chain determines the bioactivity of the molecule, with longer chained molecules being associated with increased
bioactive effects on other organisms (Adolph et al., 2003; Ceballos & Ianora, 2003). While these molecules can confer fitness benefits to diatoms, the full extent of each unique PUA’s effects and range of biological targets is still being explored (Galasso et al., 2020; Ruocco et al., 2020).

Bioactivity studies on diatom PUAs have historically focused on the invertebrate predators of diatoms, such as pelagic copepods and sea urchin larvae (Ianora et al., 2011; Koski et al., 2008; Lettieri et al., 2015; Ribalet et al., 2007; Ribalet et al., 2014; Romano et al., 2010; Ruocco et al., 2019; Varella et al., 2014; Wolfram et al., 2014). A consistent observation in these studies is that PUAs negatively affect grazer fecundity (Caldwell, 2009; Miralto et al., 1999). In a pioneering study on the effects of PUAs on copepod grazers, three specific diatom PUAs were identified as the compounds responsible for these deleterious effects: 2E,4Z,7Z-decatrienal, 2E,4Z,6Z-decatrienal, and 2E,4E-decadienal (Miralto et al., 1999). This study also highlighted a negative correlation between in situ diatom biomass and copepod reproductive success in the Adriatic Sea, suggesting that increased consumption of diatom PUAs negatively affected fecundity.

Since the identification of those three PUAs as a causative agent for reduced grazer fecundity, additional diatom PUAs have been isolated, identified, and explored for negative bioactive effects. Ianora et al. (2010) reviewed the effects of a range of diatoms on copepod fecundity and showed that, of the 21 species tested, all 21 produced negative effects on at least one of four different copepod reproductive criteria (egg production rate, hatching success, morphologically abnormal larvae, and development from naupliar stages through adulthood). In addition to the negative effects of diatom PUAs on copepod fecundity and offspring survival, antiproliferative effects on other invertebrates have also been explored. Larvae of the chordate invertebrate filter-feeder, Ciona intestinalis, developed malformed bodies in response to
dissolved PUAs (Lettieri et al., 2015). *Paracentrotus lividus* (sea urchin) embryos in the presence of 2E,4E-decadienal displayed an increased number of abnormalities and deformations as larvae (Romano et al., 2010). Furthermore, 2E,4E-decadienal caused larval development to stop at the gastrula stage and induced considerable numbers of dead or prematurely hatched urchin young.

PUAs can also have toxic effects on vertebrate cells, including cancerous cells (Martinez Andrade et al., 2018). For example, PUAs exhibited anti-proliferative activity and activated programmed cell death (apoptosis) for cancer cells originating from human adenocarcinoma colon and lung cell lines (Sansone et al., 2014). 2E,4E-decadienal also caused the mitochondria of rat liver cells to experience dysfunction and oxidative stress (Sigolo et al., 2008). These observed negative effects of PUAs on vertebrate cells suggest that PUAs may also interact with the developing planktonic offspring of marine vertebrates (Falkowksi, 2002; Nelson et al., 1995).

Marine forage fishes are a critically important vertebrate group in many marine ecosystems. They are indicators of ecosystem health, and every life history stage serves as a resource for higher-order trophic levels (Pikitch et al., 2014). Many species of forage fish spawn in nearshore environments, and their larval stages use the nearshore environment as a nursery ground to feed and develop (Pentilla, 2007). As such, they spend a good portion of their early development sympatric with nearshore diatoms, including both benthic and pelagic forms (Blaxter & Halliday, 1963; Loosanoff, 1938). Forage fishes are planktivorous throughout life, which makes them a vital link in the upward transport of nutrients in marine food chains (Cury et al. 2000, 2003; Freon et al. 2005; Bakun et al. 2010). Forage fishes are a primary food source for many different marine organisms, from sea birds and larger predatory fishes to marine mammals (Butler et al. 2010; Crawford & Dyer 1995; Jahncke et al. 2004; Logan et al. 2011; Magnussen
Economically, forage fish fisheries generate billions of dollars in revenue and are an important resource for subsistence populations in low-income regions (Palmer et al., 2018; Steiner et al., 2019).

Juvenile stages of forage fishes may be vulnerable to diatom PUAs at several points in their development. During spawning, forage fish females attach their eggs to nearshore substrates that can be heavily populated by diatoms, such as eel grass and macroalgae, moist gravel, and sand (Prazukin et al., 2022, Jewson et al., 2006; Jewson et al., 2006; Smigielski et al., 1984; Quinn et al., 2012). This spawning behavior places embryos in close proximity to benthic diatoms, providing opportunities for interaction between PUAs, developing embryos, and eventually larvae (Figure 1). The possibility for interaction with diatom PUAs is further supported by evidence that benthic diatoms are capable of PUA production at similar concentrations to that of their pelagic counterparts (Pezzolesi et al., 2017). In addition to the potential for early exposure to PUAs via benthic diatoms, another route of exposure may occur post hatch, when larvae can readily ingest pelagic phytoplankton as first-feeding larvae (Lebour, 1921; Blaxter, 1965; Purcell & Grover, 1990; Spittler et al., 1990; Friedenberg et al., 2012).

Determining whether PUAs affect the embryogenesis, development, and survival of early life-history stages of fishes is a logical next step in understanding the role of PUAs in aquatic ecosystems. As a first step toward achieving this objective, in this study I tested for the effects of PUAs on the development of a model vertebrate, the zebrafish, *Danio rerio*. Important advantages of using this model organism are the breadth of information available, their high fecundity, the ease with which they may be bred in the lab, and their rapid development (Urushibata, 2021). Although they are a tropical, freshwater fish, methods for examining zebrafish embryogenesis under toxicity are well-established (Brannen et al., 2010).
Toxicological study of zebrafish provides a straightforward way to estimate how diatom-derived PUAs may affect marine forage fish development.
METHODS

Overview

In order to test for effects of PUAs on a model aquatic vertebrate, zebrafish were exposed to a range of dissolved PUA concentrations and assessed for effects on the biometrics that I describe below. I tested hypotheses based on the following questions.

1. **Will dissolved PUAs have a negative effect on physiological processes in zebrafish larvae?**

   \( H_1 \): The average heart rate (beats per minute) of zebrafish embryos will be lower in PUA treatments when compared to a control.

   \( H_0 \): The average heart rate (beats per minute) of zebrafish embryos in PUA treatments will not be different when compared to controls.

2. **Will dissolved PUAs have a negative effect on overall zebrafish standard length, depth, and myotome height at the anus?**

   \( H_2 \): Exposure of embryonic zebrafish to dissolved PUAs will result in reductions in larval length, depth, and myotome height at the anus.

   \( H_0 \): Exposure of embryonic zebrafish to dissolved PUAs will not result in reductions in larval length, depth, and myotome height at the anus.

3. **Will dissolved PUA exposure cause increased larval zebrafish mortality?**

   \( H_3 \): Exposure of embryonic and larval zebrafish to dissolved PUAs will result in a lower probability of pre-feeding larval survival.
Exposure of embryonic and larval zebrafish to dissolved PUAs will not result in a lower probability of survival for pre-feeding larvae.

**Animal care**

AB wildtype zebrafish were maintained and bred in the lab of Dr. James Cooper at Western Washington University’s (WWU) Department of Biology. The animal care protocol for this study was approved by WWU’s Institutional Animal Welfare and Use Committee (IACUC), Bellingham, USA, in June of 2021.

**PUA treatments**

Mixtures of three PUAs, 2E,4E-decadienal (C₁₀H₁₆O, Cas No. 25152-84-5), 2E,4E-octadienal (C₈H₁₂O, Cas No. 30361-28-5; 5577-44-6), and 2E,4E-heptadienal (C₇H₁₀O, Cas No. 4313-03-5) were used to explore the effects of PUAs on zebrafish. The decision to use proportional and increasingly concentrated mixtures of these PUAs, which are heretofore referred to as heptadienal, octadienal, and decadienal, was respectively based on Ruocco et al. (2019). They found that the effects of binary and ternary combinations of these three PUAs on the development of *Paracentrotus lividus* urchin embryos to be synergistic, with negative effects increasing in exposures to combined rather than individual PUAs (Ruocco et al., 2019). This fact, and the knowledge that these three PUAs commonly co-occur *in situ*, led to the decision to use these mixtures rather than individual PUAs (Bartual et al., 2020; Vidoudez et al., 2011).

PUA mixtures were dissolved in methanol, which increases the miscibility of lipid chains (Ribalet et al., 2007; Varella et al., 2014), and homogenized in zebrafish embryo water. While
methanol is a known toxin for vertebrates at various concentrations, it has been found to be less toxic for zebrafish at low concentrations than dimethyl sulfoxide (DMSO), another solvent commonly used in toxicological and developmental work with zebrafish (Christou et al., 2020). Methanol showed no toxicity up to 1% concentration, whereas DMSO showed toxicity at or above 0.3% (Christou et al., 2020). Methanol was therefore favored over DMSO for use in this study.

Dissolved PUA mixture proportions within the range of those shown to be effective at causing developmental difficulties for urchin larvae in Ruocco et al. were used for a preliminary experiment with 24 hours post-fertilization (hpf) zebrafish embryos (decadienal 0.5 μM, heptadienal 1.0 μM, octadienal 1.5 μM; 2019) (Table 1). The proportion of decadienal added to the most concentrated mixture (high) was also within the range of decadienal concentrations shown to compromise the reproduction of the chordate invertebrate *Ciona intestinalis* (0.35, 0.4, 0.45 μg/ml; Lettieri et al., 2015). No change in mortality or morphology of zebrafish larvae was observed at any preliminary concentration after 6-days of exposure. As such, PUA mixture concentrations were increased to levels where these quantifiable effects could be observed (Table 1).

**Data collection and statistical analysis**

**Embryonic heart rate**

Embryos were assessed for heart rate changes after exposure to a range of PUA concentrations (*H₁*) (Table 1). Zebrafish embryos (25) at 24 hpf were placed in plastic petri...
dishes and constantly exposed to high, middle, and low concentrations of dissolved PUA mixtures in triplicate for 2 days (Table 1; Figure 2). Water changes were done on both days to account for PUA evaporation and to keep zebrafish waste products and bacterial and fungal levels down. For post-hoc detection of potential methanol effects, a 1% methanol control was added in addition to the embryo water control. Incubation temperatures were set at 28°C, a temperature commonly used for zebrafish rearing (Urushibata et al., 2021). At three days post-fertilization (dpf), ten randomly chosen embryos from each petri dish were video recorded for ten seconds through an Olympus SZ-CTV dissecting microscope mounted with a FLIR Blackfly USB3 Vision camera running the Micro-Manager 2.0.0 program. Heart rate beats per minute (bpm) for each embryo were determined by visually assessing contractions of the heart through the translucent chorion and epidermis. Post-measurement, live embryos were humanely euthanized via a ten-minute submersion in 0°C water before proper disposal.

To account for variance within treatments, a linear mixed model (LMM) was employed and run using R programming version 4.2.2 (lme4 and MuMin packages) to predict heart bpm under PUA treatments. REML was used to make unbiased estimates of variance and covariance parameters and nlminb optimizer to optimize a function with multiple arguments. The model also included variance introduced from petri dish replicates within treatments as a random effect. To assist with model fit, the highly variable dishes were also weighted using the varIdent function, which led to an improvement based on compared Akaike information criterion (AIC) values. For this and following morphometric LMMs, standardized parameters were obtained by fitting the model on a standardized version of the dataset. Confidence Intervals (95%) and p-values were computed using a Wald t-distribution approximation.
**Larval morphometrics**

To test hypothesis $H_2$, the same experimental setup was revisited, this time exposing embryos and larvae for 6 days (Table 1; Figure 2). This time frame allowed for 100% hatching. After a 6-day PUA incubation, larvae were humanely euthanized as described above and used in morphometric analyses. For photography, larvae were positioned laterally under a dissecting microscope in a dilute glycerol solution using LAS V4.13 from the Leica Application Suite. ImageJ was used to measure the standard length (the tip of the snout to the end of the spine), and two different dimensions of depth (deepest point of belly and myotome height at anus) of each larval photograph (Figure 3).

For statistical analysis, morphometric responses were first evaluated on an individual basis using the same R packages as for the heart rate experiment before being combined for a multivariate approach. The strength and significance of changes in each distance was determined using similar LMMs appropriate for nested designs. Following that, a principal components analysis (PCA) and permutational multivariate analysis of variance (PERMANOVA) were used to assess differences in approximate larval size between PUA treatments (R, vegan and MVN packages). The PERMANOVA was used due to the data’s departure from assumptions of multivariate normality and homogeneity (Anderson, 2017). To visualize how the approximate size of the larvae changed with each PUA increase, a PCA was performed.

Because all morphometric data were collected in one experiment, identical LMMs were used to describe all morphometric measurements (Figure 3). Also, because it had the same design as the heart rate experiment, the same LMM formula addressed the nested design and
variance needs of the data, with models containing petri dishes as a random effect, and weighted dishes leading to improved model fit based on compared AIC values (lme4 and MuMIn packages).

*Pre-feeding larval survival*

To test $H_3$, that pre-feeding larval survival will be reduced by PUA exposure, zebrafish embryos were treated with PUAs at 2 hpf. The setup mirrors the previous experiments, with the exceptions that increased numbers of replicates and embryos were included for the sake of statistical power, and total PUA concentrations were decreased for younger embryos (Table 1). Here, four replicate petri dishes were used per treatment with 30 embryos each. Throughout the experiment, larval mortalities were recorded daily until 100% mortality.

Survival probability was assessed using a Kaplan-Meier survival analysis (Jager et al., 2008). The Kaplan-Meier method is a non-parametric survival analysis also called the “time to event” approach. Here it was used to create survival probability curves for zebrafish larvae in PUAs (Di Paolo Ksenia, 2015; Jager et al., 2008). This analysis shows the probability of an event (death) at each time interval (experimental day). The calculations for each survival curve were based on the following equation, which recalibrates the probability of the event of interest at each time interval based on past occurrences (LaMorte, 2016):
\[ S_{t+1} = S_t * \left( \frac{N_t + 1 - D_t + 1}{N_t} + 1 \right) \]

\( S_{t+1} \) = the cumulative probability of surviving beyond time interval \( t \),

\( N_t \) = the number of subjects at risk at time interval \( t \),

\( D_t \) = the number of deaths during time interval \( t \)

The following analyses were carried out using the survminer package in R. First, the Kaplan-Meier equation calculated the survival probability of the zebrafish larvae in each treatment group for each day of the survival experiment, and corresponding survival curves were then generated. A log-rank test for significant differences between curves was implemented, followed by value-adjusted pairwise comparisons to clarify the significant differences between curves.
RESULTS

Heart rate analysis

The intercept for the linear mixed model of heart rate compared to all other treatments, set to the embryo water control treatment, averaged 121.2 bpm. Average larval heart rate decreased significantly by 44 bpm, a 47% reduction, in the high PUA treatment compared to the embryo water control (Table 2). The methanol control and low PUA treatments decreased average larval bpm by 14 and 15 bpm, respectively, but neither difference was found to be statistically different from the control. The explanatory power of the model without random effects, or the proportion of variance explained by the fixed effects relative to the overall variance, was 0.562 (marginal $R^2$), and was 0.949 when accounting for random effects (conditional $R^2$) (Figure 4; Table 2). This addition produced a 41% increase in the goodness of fit of the model as indicated by $R^2$.

Effects of PUA on larval zebrafish morphometrics

Standard length

The model intercept, or average larval length from the control treatment, was 3.39 mm. The effect of low PUA concentration on larval length was statistically similar to the embryo water control, with an average 0.03 mm decrease in length compared to the embryo water control. This was similar to the 0.03 mm decrease in larval length observed in the methanol only
control (Figure 5). Using this model, the effects of high and moderate PUA treatments were negative and statistically significant. The average standard length of larvae in the high treatment was 2.75 mm, 19% percent smaller than those in the embryo water control \((p = 0.003)\). The larvae in the mid PUA treatment were 17% smaller than the control, with an average length of 2.83 mm \((p = 0.006)\). When applied to standard length, the total explanatory power of the LMM was 0.88 (conditional \(R^2\)), while fixed effects alone, the PUA levels without varying dishes included (marginal \(R^2\)) explained 58% of the variation in the data (Table 3). Adding dishes as a random intercept improved the \(R^2\) fit from 0.58 to 0.88.

**Deepest vertical distance**

The average depths of larvae in the embryo water and methanol control were 0.45 and 0.43 mm, respectively. The effect of the low PUA concentration was non-significant in comparison to the control. The larvae reared in the mid PUA treatment were 23% shallower than control larvae, with an average depth of 0.35 mm (Figure 6). The average depth of larvae in the high PUA treatment was 0.32 mm, 29% percent lower than those in the embryo water control. When applied to deepest vertical distance, the total explanatory power of the LMM was 0.70 (conditional \(R^2\)), and the fixed effects alone (marginal \(R^2\)) explained 0.48 of the variation in the data (Table 4). Adding dish variation as a random effect improved the \(R^2\) measure of fit by 0.12. Within this model, the effects of high and moderate PUA treatments were negative and statistically significant \((p = 0.001; p = 0.006)\).
**Myotome height at anus**

The intercept of the model, set as average myotome height at the anus in the embryo water treatment, was 0.14 mm. The larvae in the high and mid PUA treatments were 0.15 mm on average, 8% percent larger than fish from the embryo water control. The effect of low PUA concentrations was the same as methanol, and non-significant when compared to the control (Table 5). The total explanatory power of the LMM was 0.51 (conditional $R^2$), and the fixed effects alone (marginal $R^2$) explained 11% of the variation in the data (Table 5). Adding dishes as a random intercept improved the $R^2$ fit by 0.40, indicating high variation within treatments. However, unlike the results for length and deepest vertical distance, the effects of high and moderate PUAs on myotome height were not significantly different when compared to the embryo water and methanol control treatments (Figure 7).

**Multivariate analysis of larval morphometrics**

PUA concentrations caused significant differences in larval sizes and were responsible for 65% of the overall size variation between treatment concentrations (PERMANOVA, $F_{4,322}$, $p = < 0.001$, $R^2 = 0.65$). Pairwise comparisons showed seven significantly different groups. The difference in larval sizes between the embryo water control and the methanol treated larvae was significant, indicating that 1% methanol may have a small yet statistically significant negative effect on larval zebrafish growth (Table 6). The effect of low PUAs on size was not significantly different to the effect of methanol alone. The larvae from the high and mid PUA concentrations
were significantly different in size compared to both the low PUA and methanol treatments (p = 0.01), but not significantly different from each other. Therefore, although methanol had a small effect on size (p = 0.01), the effects must be partially due to PUAs because larvae in the mid and high PUA treatments were significantly different in size to those in the methanol treatment.

A PCA of the multivariate dataset calculated 1st and 2nd principal components that accounted for 93% of the total variation in the data (~59% and ~34%, respectively) (Table 7). Ordination of PCs 1 and 2 shows the size differences between treatment groups of larvae based on the three morphological measurements used in the analysis (Figure 8). Larvae from the control, methanol, and low PUA treatments grouped closely on PC1, indicating similarity in overall size (Figure 8). Larvae from the mid and high PUA treatments were also closely associated, but spaced farther apart in length and depth, showing that not all larvae in these higher concentrations were uniformly affected. Additionally, a non-metric dimensional scaling analysis confirmed the strength of the group patterns and their positioning by converging on a similar solution after 20 iterations, with an acceptable stress value of 0.05.

*Pre-feeding survival*

Some larvae in the embryo water and methanol treatment had survivors until day 15, while larvae in low and mid PUA treatments did not live beyond day 12. Over 75% of larvae in the high PUA treatment died after 1 day of exposure, and nearly 100% had expired by day 5 (Figure 9). None of the larvae in high PUAs survived to the transition stage where yolk absorption changes to exogenous feeding (~8 dpf). A log-rank test of the survival curves indicated that significant differences in survival probabilities between the PUA treatments (p = < 0.001). Bonferroni-adjusted pairwise comparisons showed that the two control groups survived.
at similar rates (Figure 9; Table 8). The survival probability for fish in low PUAs was
significantly different when compared to either control, while the difference between fish in the
low and mid PUA concentrations were statistically non-significant. In the medium treatment, the
survival probability was significantly different compared to both controls and to the high
treatment. The highest PUA treatment had a survival probability that was significantly different
from all other treatments.
DISCUSSION

This study is the first to show that diatom specific PUAs can negatively affect the embryonic and larval development of a fish species. I found that PUAs reduced the heart rate, overall size, and pre-feeding survival of embryonic zebrafish. Findings presented here suggest that diatom derived PUAs are likely to impair the development of marine forage fishes. In the following sections I will discuss the implications of these findings on zebrafish, and assuming that observations are transferable to forage fishes, I will discuss the implications for the fitness of marine fishes exposed to PUAs during development.

Heart Rate

Zebrafish embryos exposed to higher PUA concentrations had lower heart rates than those in control treatments (Figure 4; Table 2). Embryonic fish hearts are particularly vulnerable to environmental toxins, such as PUAs, due to their highly permeable epithelia and the absence of organs responsible for blood filtration (e.g., liver and kidneys) that have yet to develop (Incardona & Scholz, 2017). Vertebrates exposed to oxylipins, such as PUAs, are known to develop atherosclerosis, experience problems with platelet aggregation and vascular constriction, and to be prone to cardiac injury (Caligiuri et al., 2017; Nayeem, 2018). Embryonic zebrafish hearts should be especially susceptible to the effects of PUA toxicity due to the ease with which these toxins can reach heart tissues and the lack of compensatory mechanisms that will arise in later
development. Therefore, it is reasonable to assume that PUAs will have similar effects on embryonic fishes and their cardiac health when they are exposed to PUAs in nature.

The consequences of early cardiac injury in fishes can be catastrophic. For example, the lake trout, *Salvelinus namaycush*, (Artedi et al., 1792) population of the Great Lakes collapsed because of heart-related issues caused by the exposure of trout embryos to the industrial pollutant dioxin (Cook et al., 2003). Chemical exposure to dioxin in lake trout caused symptoms similar to those that were seen in zebrafish exposed to PUAs. For lake trout, stress from dioxin presents first as pre-hatch heart abnormalities before progressing into problems with circulatory dysregulation, anemia, and hypoxia, eventually resulting in premature death from attendant lesions in the brain, retina, liver, and other organs (Spitsbergen et al., 1991). This circulatory dysregulation was also shown to cause yolk sac edema and impair swim bladder development (Guiney et al., 1997; Lanhametal et al., 2014; Spitsbergen et al., 1991). I observed anecdotally that zebrafish treated with PUAs frequently exhibited cardiac edema and underdeveloped swim bladders, which suggests that these fish may have experienced circulatory dysregulation symptoms beyond decreased heart rate. If the embryonic and larval stages of forage fishes are exposed to the PUA concentrations used in this study, then it is plausible that they would experience similar, life-threatening disruptions to circulatory development.

*Morphometrics*

PUA exposure affected the morphological development of zebrafish by reducing body length, body depth, and overall size (Figure 5-8; Table 3-6). If the mechanisms which retard zebrafish growth after PUA exposure are similar in forage fishes, then PUA exposure may affect
forage fish fitness through multiple pathways. For example, larval fish survival is coupled with larval body size, with smaller larvae experiencing higher rates of predation (Cowan et al., 1996; Paradis, 1996). For any planktonic organism the risk of predation is significant, and this is also true for forage fishes, where predation is one of the most important causes of mortality (Miller et al., 1988; Bailey & Houde, 1989; Paradis, 1996; Peterson & Wroblewski, 1984). As such, high selective pressure exists on planktonic organisms, including forage fishes, to grow rapidly. If through PUA exposure causes forage fishes to grow at a slower rate, then this increases their exposure to predation which can eventually affect recruitment success (Houde, 2008).

Smaller fishes also tend to have slower swimming speeds and lower feeding efficiencies in comparison to larger conspecifics (Houde, 2008; Gleason & Bangston., 1996; Houde & Schekter, 1980; Hare & Cowen, 1997). In bay anchovies (Anchoa mitchilli; Valenciennes, 1848) feeding efficiency has been shown to increase with size (Gleason & Bangston., 1996; Houde & Schekter, 1980). In bluefish (Pomatomus saltatrix; Linnaeus, 1766) larger size was correlated with higher survival rates from yolk-sac-stage embryos to first-feeding larvae (Hare & Cowen, 1997). Raventos and MacPherson (2005) also found that larval size was an accurate predictor of post-settlement survival for two species of reef fishes, the five-spotted wrasse (Symphodus roissali; Risso, 1810) and the ocellated wrasse (Symphodus ocellatus; Linnaeus, 1758).

The observation that fish larvae experience slower swimming speeds and reduced feeding efficiencies relative to later life stages is likely a result of the relationship between larval size and fluid dynamics (Van Leeuwen et al., 2015; Voesenek et al., 2018). After hatching most larval fishes continue to develop by way of yolk and lipid reserves provided by their mothers (Kamler, 2008). They quickly develop functional mouth parts, allowing them to feed and acquire energy exogenously (Yúfera & Darias, 2007).
Larval stages of fishes, including forage fishes, encompass a size range that places them in an intermediate hydrodynamic regime between laminar (viscous) and turbulent (inertial) flow (Voesenek et al., 2018). The relative dominance of viscous and inertial forces is estimated by calculating the Reynolds number (Van Leeuwen et al., 2015). Larval sizes < 5mm, which encompasses the sizes of zebrafish measured here, and those of most larval forage fishes, places them in a low Reynolds number environment in which viscous forces dominate (Van Leeuwen et al., 2015). Because movement through more viscous fluids requires a higher cost of transport (Schmidt-Nielson, 1972), and because higher viscosities lower maximum swimming speeds and reduce a larva’s ability to avoid predators, prolonging the time that young fishes spend in a low Reynolds number environment can greatly reduce survival (Voesenek et al., 2018). Further, it is a taxation on endogenous energy reserves. If these reserves are exhausted before transition to exogenous feeding, starvation is likely to result (Yin & Blaxter, 1987).

Existing in a high viscosity environment also requires small larval fishes to employ higher forces (relative to body size) when suction feeding, which is usually the only feeding strategy available for them (China & Holzman, 2014; Cooper et al., 2020). The ratio of energy expended to energy acquired during feeding will therefore be higher for smaller fishes. Additionally, the movement required by small fish larvae in viscous environments requires the beating of their tails at a high frequency, which in turn is dependent on the use of fast-twitch muscles. These muscles are fueled by mitochondria-dense fibers with a high ATP demand and energy reserves (Huriaux et al., 1996; Van Leeuwen et al., 2016). Prolonged residence in a high-viscosity environment increases the likelihood of starvation for larval fishes, which is already the largest source of mortality for most larval fishes (Hjort, 1914; Houde, 2002).
**Pre-feeding survival**

In experiments where pre-feeding larvae were exposed to PUAs and reared until starvation, PUA exposure accelerated rates of larval mortality in comparison to control treatment larvae (Figure 9; Table 8). Most specimens exposed to high PUA concentrations did not survive until they would otherwise have begun to feed exogenously, and mortality rates were higher for fish from the low and medium PUA treatments in comparison to the controls (Figure 9; Table 8). The inference that PUAs impose general stress to developing fishes resulting in reductions in survival is supported by my finding that PUAs affected zebrafish embryonic heart rates, and by extension circulatory dysregulation and the associated damaging effects (Caligiuri et al., 2017; Nayeem, 2018). The implications of this finding are significant for the ecology of forage fish larvae. The added physiological stress imposed by PUA exposure may exacerbate starvation stress and reduce survival in larval forage fishes.

**Conclusions and future directions**

This study provides the first evidence that diatom derived PUAs can negatively affect the development of fishes. This is also the first study to explore the effects of PUAs on a vertebrate. Given the sympatry of diatoms with embryonic and larval stages of forage fishes in most temperate marine ecosystems, and the clear effects of PUAs on a larval fish, the likelihood that PUAs will negatively affect the fitness of forages fishes seems high. If true, then this pathway represents an unexplored mechanism that might explain some of the variation in interannual variability in forage fish population dynamics. My findings also suggest that there are pathways
for diatom-derived PUAs to affect marine food webs and marine secondary production beyond the diatom-copepod link.

While not quantified in this study, zebrafish embryos in the size and mortality experiments tended to hatch later if they had been exposed to high PUA concentrations, and many needed assistance (manually rupturing the chorion) in order to be released from their eggs. These observations suggest that the hatching success of embryos born in areas of high diatom density might be compromised. As such, hatching success might be a useful metric for quantifying the effects of PUAs on the development of fish species. My findings also suggest that future experiments could include other meaningful markers of PUA stress on fitness like the number of edematous larvae, swim bladder development, and later life history stages. Given the clear potential presented here for negative interactions between PUAs and developing fish, especially forage fishes, further exploration of the effects caused by PUA exposure will likely add to our current understanding of the nearshore environmental ecology of forage fishes and diatom PUAs and its implications.
Figure 1. Pathways for embryonic and larval forage fish interactions with PUAs. A) A forage fish nearshore spawning event where eggs are deposited on substrate harboring benthic diatoms. B) Embryos experiencing PUA exposure from benthic diatoms releasing PUAs upon grazing-induced damage to diatom cell walls. C) Early-stage larval forage fish maturing near hatch location with continued exposure to dissolved PUAs. D) First-feeding larval forage fish experiencing PUA exposure indirectly through pelagic zooplankton grazing and directly via ingestion of diatoms and zooplankton.
Table 1. Total PUA mixture concentrations (µg/ml) and individual PUA proportions (amount added) for all experiments (Total = total PUAs in µg/ml).

<table>
<thead>
<tr>
<th>PUA Mixture Concentrations and Composition (µg/ml)</th>
<th>Preliminary Experiment</th>
<th>PUA</th>
<th>Low</th>
<th>Mid</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount added:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octadienal</td>
<td>0.12</td>
<td>0.56</td>
<td>1.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heptadienal</td>
<td>0.06</td>
<td>0.33</td>
<td>0.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decadienal</td>
<td>0.04</td>
<td>0.19</td>
<td>0.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.22</td>
<td>1.08</td>
<td>2.16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Morphometrics and Heart Rate Experiments</th>
<th>PUA</th>
<th>Low</th>
<th>Mid</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octadienal</td>
<td>1.12</td>
<td>5.59</td>
<td>11.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heptadienal</td>
<td>0.66</td>
<td>3.30</td>
<td>6.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decadienal</td>
<td>0.38</td>
<td>1.90</td>
<td>3.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>2.16</td>
<td>10.80</td>
<td>21.59</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Survival Experiment</th>
<th>PUA</th>
<th>Low</th>
<th>Mid</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octadienal</td>
<td>1.12</td>
<td>2.24</td>
<td>3.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heptadienal</td>
<td>0.66</td>
<td>1.32</td>
<td>1.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decadienal</td>
<td>0.38</td>
<td>0.76</td>
<td>1.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>2.16</td>
<td>3.24</td>
<td>6.48</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2. Design for 3 nested developmental experiments to test for PUA effects on zebrafish larvae. Blue circles represent petri dishes housed in an environmental incubator. Control = embryo water control; Methanol = embryo water plus methanol solvent. Larval morphometrics, 25 embryos per plate, top right; Heart rate, 25 embryos per plate, top left (red * = dish expired before recording); Survival, 30 embryos per plate, bottom.

Figure 3. The three dimensions measured for larval fish morphometrics: Line 1 = standard length; Line 2 = deepest vertical point; Line 3 = myotome height at anus (Image Credit: Lizzy Griffiths)
Table 2. Linear mixed model results for heart rate in beats per minute (Bpm) of 3 dpf zebrafish larvae after constant PUA exposure at 2 different concentrations (Table 1). (Intercept) = Control treatment (R, lme4, sjPlot and MuMIn packages). Bolded values indicate statistically significant p-values.

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Bpm Estimates</th>
<th>CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Control] (Intercept)</td>
<td>121.20</td>
<td>106.83 – 135.57</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treatment [Methanol]</td>
<td>-15.00</td>
<td>-39.22 – 9.22</td>
<td>0.187</td>
</tr>
<tr>
<td>Treatment [Low]</td>
<td>-14.20</td>
<td>-38.42 – 10.02</td>
<td>0.208</td>
</tr>
<tr>
<td>Treatment [High]</td>
<td>-44.10</td>
<td>-71.18 – -17.02</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Random Effects

- $\sigma^2$: 141.71
- $\tau_{00}$ DishNumber: 143.26
- $N_{DishNumber}$: 11

Observations: 110

Marginal $R^2$/Conditional $R^2$: 0.562/0.949
Figure 4. Larval heart beats per minute under PUA exposure. PUA treatments are differentiated by color. Petri dishes are labeled 1-3 on the x-axis and have letters and numbers representing their treatments and replicates (Figure 2). Blue bars indicate treatment averages and black bars represent dish averages. Dishes A1-3 and plate B3 expired on day 1 and data are not available.
Figure 5. Larval standard length under PUA exposure. PUA concentrations are differentiated by color. Length is in mm on the y-axis. Individual replicate petri dishes for each treatment are labeled A1-E3 on the x-axis with letters representing concentration (A = High, B = Mid, C = Low, D = Methanol, E = Control). Blue bars indicate treatment averages and black bars within dish averages.
Table 3. Linear mixed model results for standard length (Length) of zebrafish 7 dpf larvae after 6 days of constant PUA exposure at 3 different concentrations (Table 1). All groups were compared to the intercept, which in this model is the average from the control treatment. Bolded values indicate statistically significant p-values (R, lme4, sjPlot and MuMIn packages).

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Estimates</th>
<th>CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Control] (Intercept)</td>
<td>3.39</td>
<td>3.16 – 3.62</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treatment [Methanol]</td>
<td>-0.03</td>
<td>-0.40 – 0.33</td>
<td>0.841</td>
</tr>
<tr>
<td>Treatment [Low]</td>
<td>-0.03</td>
<td>-0.40 – 0.33</td>
<td>0.851</td>
</tr>
<tr>
<td>Treatment [Mid]</td>
<td>-0.57</td>
<td>-0.93 – -0.20</td>
<td><strong>0.006</strong></td>
</tr>
<tr>
<td>Treatment [High]</td>
<td>-0.64</td>
<td>-1.00 – -0.27</td>
<td><strong>0.003</strong></td>
</tr>
</tbody>
</table>

**Random Effects**

- $\sigma^2$: 0.02
- $\tau_{00}$ DishNumber: 0.04
- ICC: 0.71
- N DishNumber: 15

- Observations: 326
- Marginal R$^2$ / Conditional R$^2$: 0.585 / 0.879
Figure 6. Deepest vertical point (Figure 3; Depth 2) for zebrafish larvae exposed to PUAs. PUA concentrations are differentiated by color. Depth is in mm on the y-axis. Petri dishes are labeled A1-E3 on the x-axis and have letters and numbers representing their concentration and replicates (A = High, B = Mid, C = Low, D = Methanol, E = Control). Blue bars indicate group averages and black bars within dish averages.
Table 4. Linear mixed model results for deepest vertical distance (Depth 2) of 7 dpf zebrafish larvae after constant PUA exposure at 3 different levels (Table 1). All groups were compared to the intercept, which in this model the control treatment (R, lme4, sjPlot and MuMIn packages).

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Estimates</th>
<th>CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Control] (Intercept)</td>
<td>0.45</td>
<td>0.41 – 0.49</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treatment [Methanol]</td>
<td>-0.02</td>
<td>-0.08 – 0.04</td>
<td>0.463</td>
</tr>
<tr>
<td>Treatment [Low]</td>
<td>-0.02</td>
<td>-0.08 – 0.04</td>
<td>0.471</td>
</tr>
<tr>
<td>Treatment [Mid]</td>
<td>-0.10</td>
<td>-0.16 – -0.04</td>
<td>0.006</td>
</tr>
<tr>
<td>Treatment [High]</td>
<td>-0.13</td>
<td>-0.19 – -0.06</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Random Effects

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(\sigma^2)</td>
<td>0.00</td>
</tr>
<tr>
<td>(\tau_{00}) DishNumber</td>
<td>0.00</td>
</tr>
<tr>
<td>ICC</td>
<td>0.43</td>
</tr>
<tr>
<td>N DishNumber</td>
<td>15</td>
</tr>
</tbody>
</table>

| Observations | 326  |
| Marginal R\(^2\) / Conditional R\(^2\) | 0.477 / 0.701 |
Figure 7. Myotome height at the anus (Depth 3). PUA levels are differentiated by color. Measurements are in mm on the y-axis. Petri dishes are labeled A1-E3 on the x-axis. Letters and numbers represent concentration and replicate (A = High, B = Mid, C = Low, D = Methanol, E = Control). Blue bars indicate group average and black bars within dish average.
Table 5. Linear mixed model results for myotome height at the anus (Depth 3) of 7 dpf zebrafish larvae after constant PUA exposure at 3 different concentration levels (Table 1). All groups were compared to the intercept, which in this model is the control treatment. Bolded values indicate statistically significant p-values (R, lme4, sjPlot and MuMIn packages).

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Estimates</th>
<th>CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Intercept)</td>
<td>0.14</td>
<td>0.12 – 0.15</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treatment [Methanol]</td>
<td>-0.01</td>
<td>-0.04 – 0.02</td>
<td>0.402</td>
</tr>
<tr>
<td>Treatment [Low]</td>
<td>-0.01</td>
<td>-0.03 – 0.02</td>
<td>0.671</td>
</tr>
<tr>
<td>Treatment [Mid]</td>
<td>0.01</td>
<td>-0.02 – 0.03</td>
<td>0.466</td>
</tr>
<tr>
<td>Treatment [High]</td>
<td>0.01</td>
<td>-0.02 – 0.03</td>
<td>0.526</td>
</tr>
</tbody>
</table>

**Random Effects**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>( \sigma^2 )</td>
<td>0.00</td>
</tr>
<tr>
<td>( \tau_{00 \text{ DishNumber}} )</td>
<td>0.00</td>
</tr>
<tr>
<td>ICC</td>
<td>0.45</td>
</tr>
<tr>
<td>N \text{ DishNumber}</td>
<td>15</td>
</tr>
</tbody>
</table>

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Observations</td>
<td>326</td>
</tr>
<tr>
<td>Marginal ( R^2 ) / Conditional ( R^2 )</td>
<td>0.110 / 0.506</td>
</tr>
</tbody>
</table>
Table 6. PERMANOVA pairwise comparisons with non-adjusted and Bon Ferroni adjusted p-values. Sig = p < 0.05 (R, pairwiseAdonis and vegan packages).

<table>
<thead>
<tr>
<th>Pair</th>
<th>Df</th>
<th>SS</th>
<th>F Model</th>
<th>R2</th>
<th>p-value</th>
<th>p-adjusted</th>
<th>Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>High vs Mid</td>
<td>1.000</td>
<td>0.009</td>
<td>2.674</td>
<td>0.022</td>
<td>0.083</td>
<td>0.830</td>
<td></td>
</tr>
<tr>
<td>High vs Low</td>
<td>1.000</td>
<td>0.349</td>
<td>186.363</td>
<td>0.593</td>
<td>0.001</td>
<td>0.010</td>
<td>*</td>
</tr>
<tr>
<td>High vs Methanol</td>
<td>1.000</td>
<td>0.343</td>
<td>184.448</td>
<td>0.596</td>
<td>0.001</td>
<td>0.010</td>
<td>*</td>
</tr>
<tr>
<td>High vs Control</td>
<td>1.000</td>
<td>0.393</td>
<td>221.308</td>
<td>0.634</td>
<td>0.001</td>
<td>0.010</td>
<td>*</td>
</tr>
<tr>
<td>Mid vs Low</td>
<td>1.000</td>
<td>0.243</td>
<td>134.487</td>
<td>0.512</td>
<td>0.001</td>
<td>0.010</td>
<td>*</td>
</tr>
<tr>
<td>Mid vs Methanol</td>
<td>1.000</td>
<td>0.237</td>
<td>132.489</td>
<td>0.515</td>
<td>0.001</td>
<td>0.010</td>
<td>*</td>
</tr>
<tr>
<td>Mid vs Control</td>
<td>1.000</td>
<td>0.279</td>
<td>163.046</td>
<td>0.560</td>
<td>0.001</td>
<td>0.010</td>
<td>*</td>
</tr>
<tr>
<td>Low vs Methanol</td>
<td>1.000</td>
<td>0.000</td>
<td>0.288</td>
<td>0.002</td>
<td>0.755</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Low vs Control</td>
<td>1.000</td>
<td>0.002</td>
<td>5.014</td>
<td>0.035</td>
<td>0.011</td>
<td>0.110</td>
<td></td>
</tr>
<tr>
<td>Methanol vs Control</td>
<td>1.000</td>
<td>0.003</td>
<td>9.461</td>
<td>0.065</td>
<td>0.001</td>
<td>0.010</td>
<td>*</td>
</tr>
</tbody>
</table>

Table 7. Standard deviation, proportion of variance, and cumulative proportion explained by principal components 1 (PC1), 2 (PC2) and 3 (PC3).

<table>
<thead>
<tr>
<th></th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard deviation</td>
<td>1.3302</td>
<td>1.0062</td>
<td>0.467</td>
</tr>
<tr>
<td>Proportion of Variance</td>
<td>0.5898</td>
<td>0.3375</td>
<td>0.0727</td>
</tr>
<tr>
<td>Cumulative Proportion</td>
<td>0.5898</td>
<td>0.9273</td>
<td>1.0000</td>
</tr>
</tbody>
</table>
Figure 8. PCA of the variation in size of fish larvae based on PC1 and PC2. Vectors in blue show the influence of the variable on the position of larvae. PC1 increases with Length (standard length) and Depth 2 (deepest point) vectors. Depth 3 (myotome height at anus) increased on PC2. Larger larvae are located toward the top and right and smaller to the bottom left (R, plotly package).
Figure 9. Kaplan-Meier survival probability curves. The y-axis shows the probability of survival as a ratio from 0-1. Three increasing concentrations of PUAs are shown by color, with 2 controls (Control = untreated embryo water; Methanol = 1% methanol solvent).

Table 8. P-values from Bonferroni adjusted pairwise comparisons of 5 different treatment group survival curves in the Kaplan-Meier analysis.

<table>
<thead>
<tr>
<th>PUA</th>
<th>Low</th>
<th>Mid</th>
<th>High</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mid</td>
<td>0.1742</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>High</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>0.0028</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>-</td>
</tr>
<tr>
<td>Methanol</td>
<td>0.0076</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.5976</td>
</tr>
</tbody>
</table>
Works Cited

https://doi.org/10.1016/S0040-4020(03)00382-X

https://doi.org/10.1002/9781118445112.stat07841

http://archive.org/details/petriartedisueci03arte

https://doi.org/10.1016/S0065-2881(08)60187-X

https://doi.org/10.1007/s11160-009-9118-1

https://doi.org/10.3390/md14010023


https://doi.org/10.1073/pnas.1323205111


https://doi.org/10.1016/j.scitotenv.2019.134490


https://doi.org/10.1111/j.1474-919X.1995.tb08029.x


https://doi.org/10.1006/jmsc.2000.0712


https://doi.org/10.1079/9780851996332.0103

https://gallica.bnf.fr/ark:/12148/bpt6k6365342h


https://doi.org/10.1016/j.aquatox.2015.10.014


https://doi.org/10.1126/science.281.5374.237


Hjort, J. (1914). Fluctuations in the great fisheries of northern Europe viewed in the light of biological research. ICES.

Houde, E. D. (2008). Emerging from Hjort’s Shadow. *Journal of Northwest Atlantic Fishery Science, 41,* 53–70. [https://doi.org/10.2960/J.v41.m634](https://doi.org/10.2960/J.v41.m634)


Linnaeus, C. (1789). *Systema naturae per regna tria naturae, secundum classes, ordines, genera, species; cum characteribus, differentiis, synonymis, locis. apud J.B. Delamolliere.*


Pondaven, P. (2018). Influence of diatom diversity on the ocean biological carbon pump. *Nature Geoscience, 11*(1), 27–37. [https://doi.org/10.1038/s41561-017-0028-x](https://doi.org/10.1038/s41561-017-0028-x)


