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Polyunsaturated Aldehyde Production in the Salish Sea: A Survey of Benthic Diatom Producers and the Influence of Coastal Upwelling

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By

Jeremy Johnson

Accepted in Partial Completion
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Master's Thesis

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Jeremy Johnson

May 18th, 2023

Polyunsaturated Aldehyde Production in the Salish Sea: A Survey of Benthic Diatom Producers and the Influence of Coastal Upwelling

A Thesis
Presented to
The Faculty of
Western Washington University

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

By Jeremy Johnson
May 2023

Abstract

In coastal, nutrient-rich waters like the Salish Sea, diatoms dominate the phytoplankton community during seasonal upwelling events. Diatoms were once believed to be an ideal food source for primary consumers like copepods, but their role in food web dynamics changed upon discovery that diatoms can produce organic compounds upon cell death known as polyunsaturated aldehydes (PUAs). These compounds directly affect the reproductive success of diatom consumers by reducing egg production and viability, deforming embryos, and delaying embryonic development. PUA production dynamics have been assessed under varying nutrient concentrations and culture age, but no study has tested the effect of elevated dissolved CO_2 (pCO_2) on the production of these organic molecules. In addition, most surveys of PUA-producing diatoms have only assessed pelagic production. In this study, I isolated benthic diatom species from the Salish Sea and tested them for PUA production. I also conducted a microcosm upwelling experiment using a benthic and pelagic diatom under varying pCO_2 levels to assess whether PUA production is affected by inorganic carbon level.

I found that all Salish Sea benthic diatom species tested produced PUA molecules, and elevated pCO_2 did not significantly affect the production of PUA molecules. Experimental results indicate that upwelling events alone are likely not causing an increase in PUA production. The survey results indicate that benthic diatoms should be included in future PUA producer surveys to assess PUA production in the water column. Future research should assess the synergy between elevated pCO_2 and nutrient limitation on PUA production, as these conditions are expected with worsening climate change. In addition, future studies on the impact of PUAs on grazer fecundity should be expanded to include benthic diatom consumers, including mollusks and other benthic invertebrates.

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Introduction

Diatoms and their Global Influence

Diatoms are a class of microscopic, unicellular algae that dominate coastal, nutrient-rich waters like the Salish Sea (Nelson et al. 1995, Armbrust et al. 2009, Belluz et al. 2021). These algae are highly efficient photosynthesizers, contributing an estimated 40% of total primary production in the ocean and 20% of oxygen production globally (Mann 1999, Falkowski et al. 2004, Harvey et al. 2019). The photosynthetic efficiency of diatoms is heightened in coastal waters, where diatoms contribute up to 70% of net primary production (Uitz et al. 2010). This primary production, to a large extent, serves as the base of the marine food web and supports higher trophic level organisms, like commercially important forage fish and benthic invertebrates (Coutteau 1996, Kumaran et al. 2017, Kaparapu 2018).

Diatoms are cosmopolitan, inhabiting both the pelagic and benthic environments of the photic zone (Cahoon 1999, Kamp et al. 2011, Leynaert et al. 2018). Pelagic diatoms inhabit the sunlit layers of the water column and are a primary resource for zooplankton, specifically copepods (Clarke et al. 1939, Wiggert 2005, Liu et al. 2016). These diatoms are also major contributors to the biological pump, whereby organically fixed carbon sinks to the bottom of the ocean and is buried in marine sediment through sinking fecal pellets and marine snow (Agusti et al. 2015, Benoiston et al. 2017). In contrast, benthic diatoms inhabit the seafloor of the photic zone, and secrete polysaccharides that allow them to adhere to sediment of all grain sizes and other organisms like eelgrass and macroalgae (Jonge 1985, Tong & Derek 2021). While they don’t contribute as significantly to nutrient cycling and the biological pump as their pelagic counterparts, benthic diatoms are ubiquitous in near-shore locations and can support entire food chains of larger, higher trophic level organisms (Christianen et al. 2017). Pelagic diatoms are

well-studied given their importance as a resource to higher trophic levels (Taylor et al. 2007). Benthic diatoms, in contrast, are comparatively understudied (Althouse et al. 2014).

The cosmopolitan nature of diatoms is linked to their metabolic flexibility, photosynthetic efficiency, and grazing defense properties (Hamm et al. 2003, Roberts et al. 2007, Lepetit et al. 2017, Bondoc et al. 2018). In coastal locations, diatom blooms are initiated during seasonal upwelling events, which increase surface concentrations of essential nutrients like nitrate and carbon dioxide (CO_2) (Fawcett & Ward 2011). However, when nitrate concentrations decrease, diatoms can sustain high growth rates using other nitrogen sources (Rogato et al. 2016). For example, diatoms can use and grow rapidly on recycled nitrogen like urea and ammonium (da Silva et al. 2009). They can also use multiple forms of dissolved inorganic carbon (DIC) for carbon fixation, including CO_2 and bicarbonate, and can use these molecules at similar rates (Tortell et al. 1997, Trimborn et al. 2008).

Diatoms also have a high photosynthetic efficiency. Like all photoautotrophs, they use the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) in the Calvin cycle to fix CO_2 into chemical energy during the process of photosynthesis (Young et al. 2016, Sethi et al. 2020). RubisCO evolved at a time when atmospheric CO_2 concentrations were much higher than current levels (Nisbet et al. 2007). Depending on temperature and CO_2:O_2 ratios, this disparity in CO_2 concentration can cause RubisCO to occasionally catalyze a competing reaction, the oxygenase pathway (Young & Hopkinson 2017). In this photorespiratory reaction, molecular oxygen is taken up and carbon molecules are broken down, yielding CO_2 (Young & Hopkinson 2017, Cummins 2021). Diatoms have the most efficient form of RubisCO known, which allows for high photosynthetic efficiency and less photorespiration (Roberts et al. 2007). In addition,

diatoms have various carbon-concentrating mechanisms (CCMs) that allow for increased efficiency of RubisCO in carbon-poor environments (Young et al. 2016, Matsuda et al. 2017).

In addition to mechanisms that select for high or sustained growth and photosynthetic rates under environmental variability, diatoms have also evolved phenotypes to minimize grazing mortality. Diatoms biomanufacture siliceous frustules that surround the cell membrane and can inhibit predation (Sumper & Brunner 2005, Reid et al. 2021). For example, frustules with a higher silica content are less palatable to predators than those with less silica (Bondoc et al. 2018). In addition, some diatoms produce needle-like spines on their frustules, which create a physical boundary between the diatoms and their predators (Hamm et al. 2003). Many diatom species also excrete polysaccharides through pores in the silica. These polysaccharides can link diatoms together to form multicellular chains and allow for gliding, substrate adhesion, and predator deterrence, as smaller grazers may be unable to consume large chains of cells (Malej & Harris 1993, Tolhurst 2008, Bondoc et al. 2018).

An Introduction to Polyunsaturated Aldehydes

Due to their high abundance across disparate marine ecosystems and their prevalence in zooplankton fecal pellets, diatoms were once considered optimal food for their pelagic consumers, like copepods (Legendre 1990, Arendt et al. 2005). This paradigm shifted, however, when it was found that copepod egg production and viability decreased and embryo malformation increased when adult females fed on diatom diets (Ban et al. 1997, Ianora et al. 1999, Ianora et al. 2004). Following this discovery, researchers identified polyunsaturated aldehydes (PUAs) to be the causative agent of this reduced fecundity. This effect resulted from the presence of PUAs in copepod gonads during egg development, when lipid requirement is high (Miralto et al. 1999). Subsequent studies have shown that PUAs impact sea urchin larvae by

impeding fertilization, deforming embryos, and arresting embryonic development (Caldwell et al. 2002, Caldwell et al. 2004, Romano et al. 2010, Varrella et al. 2014, Ruocco et al. 2018).

PUAs are a class of oxylipins produced by many pelagic and benthic diatoms (Wichard et al. 2005a, Vidoudez et al. 2011a, Vidoudez et al. 2011b, Pezzolesi et al. 2017). PUAs are characterized by their aldehyde functional group and long, unsaturated carbon tail (Figure 1). Diatoms can synthesize a variety of PUAs of varying chain length, degree of unsaturation, and isometric conformation (Figure 1, Wichard et al. 2005a, Jüttner et al. 2010, Vidoudez et al. 2011b, Lixia et al. 2019). PUA synthesis is heavily enzyme-dependent and tightly controlled within diatoms. Synthesis of particulate PUAs occurs during damage to the cell membrane from predator grazing or apoptosis (Orefice et al. 2022). Upon cell death, polyunsaturated fatty acids like eicosapentaenoic and hexadecatrienoic acid are released from the cell and chloroplast membranes (Taylor et al. 2009). These lipids are oxidized by the enzyme lipoxygenase to form hydroperoxides and, later, PUAs (Orefice et al. 2022). Diatoms can also release dissolved PUAs into the water column without membrane disruption (Vidoudez & Pohnert 2008).

In addition to reducing predator fecundity, PUAs have other ecological roles. Dissolved PUAs can act as information chemicals by signaling cellular autolysing in surrounding diatoms (Vidoudez & Pohnert 2008, Ribalet et al. 2014, Franze et al. 2018). PUA production is observed to be highest during bloom die-off, and their ability to trigger autolysing suggests PUAs may act as a signal to limit the time span and biomass of diatom blooms (Vidoudez & Pohnert 2008, Ribalet et al. 2014, Franze et al. 2018). By limiting the duration and biomass of their blooms, diatoms can control their nutrient usage in a local environment and, possibly, control predator populations by reducing the amount of primary production available for secondary production.

Knowledge Gap and Research Questions

To date, studies on PUAs are largely restricted to pelagic diatoms, with comparatively little known about PUA production in benthic diatoms. One study from the Adriatic Sea showed that three abundant species of benthic diatoms produced PUAs (Pezzolesi et al. 2017). While the Salish Sea harbors pelagic PUA-producing diatoms like *Skeletonema* spp., the presence of benthic PUA-producers has not been explored (Wichard et al. 2005a, Paul et al. 2010). Given that PUAs can reduce both pelagic and benthic predator fecundity, it is imperative that we gain an understanding of benthic PUA production to form a more holistic view of their role in predator-prey dynamics across disparate marine environments.

In addition, no research has been done on the link between PUA production and environmental CO_2 level (pCO_2), which changes frequently in coastal areas due to upwelling and vertical mixing associated with diel tidal cycles (Rippeth et al. 2014, Cai et al. 2020). During upwelling events, carbon- and nutrient-rich water from depth replenishes surface water moved offshore during Eckman transport (Huyer 1983). This phenomenon, combined with favorable light and temperature conditions, causes diatom blooms each spring and summer (Montero et al. 2017, Spilling et al. 2018). While ambient pCO_2 averages about 400 µatm in the global ocean, coastal upwelling in the Pacific Northwest can introduce CO_2-enriched water with partial pressures as high as 1200 µatm (Haigh et al. 2015, Evans et al. 2021). Many species of phytoplankton respond favorably to carbon enrichment, likely a result of the poor affinity of pCO_2 binding in RubisCO (Bach et al. 2019, Sethi et al. 2020). Increased pCO_2 levels may alleviate inorganic carbon stress in diatoms and lead to increased carbon fixation and secondary metabolite production (Harvey et al. 2019). Carbon-rich PUA precursor molecules may be a repository for excess carbon produced during these high DIC upwelling events. In turn, this

could lead to increased PUA production. Establishing if there is a correlation between CO_2-enrichment and PUA production will inform whether upwelling-induced diatom blooms are a repository for PUA-enriched cells.

The purpose of this study was to 1) survey Salish Sea benthic diatoms for the production of PUAs, and 2) test the effect of elevated dissolved carbon dioxide (pCO_2) concentrations on PUA production in both a pelagic and benthic diatom. The PUAs chosen for this analysis were 2,4-heptadienal (heptadienal), 2,4-octadienal (octadienal), and 2,4-decadienal (decadienal) due to their proven bioactivity, or ability to affect predator fecundity (Jüttner et al. 2010).

Methods

Study Site and Model Organisms

This research focused on pelagic and benthic diatom species residing in the Salish Sea. The pelagic diatom species, *Skeletonema marinoi*, is a known PUA producer and was purchased from the National Center for Marine Algae and Microbiota. Benthic diatom species were isolated from the Salish Sea.

Benthic Diatom Sampling, Isolation, and Identifications

Samples for benthic diatom isolation were collected during low tide events at Marine Park in Bellingham, Washington (48.7193° N, 122.5158° W) and at Shannon Point Marine Center sea tables in Anacortes, Washington (48.50805° N, 122.68427° W). Marine Park is a flat, cobble beach with eelgrass beds, and benthic diatom assemblages were collected from eelgrass blades. At the lab, individual diatoms were isolated using a micropipettor and washed several times using 9-well wash plates. Once isolated, diatoms were placed into 125 mL polycarbonate bottles with autoclaved filtered seawater (AFSW) amended with F/4 growth medium. Cultures were routinely assessed for purity. Diatom species were visualized using a combination of dissecting, compound, and scanning electron microscopy (SEM), and species identified by cross-referencing with published sources.

Eight benthic diatom species were isolated and identified from the Salish Sea. Identified species were *Fragilariopsis pseudonana*, *Licmophora communis*, *Licmophora flabellata*, *Cylindrotheca closterium*, *Fistulifera saprophila*, *Nitzschia* sp. a, *Nitzschia* sp. b, and *Navicula* sp. (Ehrenberg 1839, Hassle 1965, Lobban et al. 2011, Zgrundo et al. 2013, Kociolek et al. 2023, Figure 3). A minor contamination in the *C. closterium* culture was found during SEM imaging,

and the contaminant identified as *Navicula vara* (Witkowski et al. 2000, Figure 3e). No SEM images were taken for *Licmophora communis*.

Culture Maintenance

Diatom cultures were maintained in 125 mL polycarbonate bottles using AFSW amended with F/4 growth medium. Cultures were grown in environmental incubators on a 11:13 L:D cycle at 15°C. Once per week, cultures were homogenized by gentle mixing, reduced by 75%, and refilled with growth medium. For some benthic species, new polycarbonate bottles were used each week due to colonies sticking to the bottom of bottles.

Benthic Diatom Survey

Post-isolation, benthic diatoms were dispersed into triplicate glass bowls covered with saran wrap to minimize evaporation and filled with 200 mL AFSW amended with F/4 growth medium. After 6 days, each culture was filtered (10 mL) for chlorophyll quantification. The remaining volume (~190 mL) was filtered for PUA measurements, processed, and run on GC-MS for quantification. Each set of triplicate cultures were averaged for chlorophyll-*a* level and PUA production.

Upwelling Experimental Design

To test for the effect of CO_2-enrichment on PUA production, a sealed bottle design was selected. This design was chosen as a method to replicate an isolated upwelling event in a subsurface water sample that is spatially isolated from the surface where air-sea gas exchange could occur (Figure 4).

The CO_2-enrichment experiment was performed using the ocean acidification system described in Love et al. (2017). During experiments, the 400 µatm pCO_2 level was chosen to

reflect present day ambient pCO_2 (Riebesell et al. 2007). Elevated DIC levels (800 and 1200 µatm) indicative of upwelling events were chosen based on Feely et al. (2008). The ambient pCO_2 served as a control, while the other treatments represented DIC-rich water caused by upwelling. To create desired pCO_2 levels, carbon-scrubbed ambient air was mixed with research grade CO_2 gas (99% CO_2, AirGas) in known concentrations using mass flow controllers. This gas mixture was bubbled into carboys containing AFSW amended with F/4 growth medium for at least 24 hours and is referred to as pre-equilibrated media.

Within each carbon dioxide level, 500 mL of pre-equilibrated media was inoculated with 5 mL of dense diatom cultures (174.54 µg chlorophyll-*a*/L for *Skeletonema marinoi* and 398.61 µg chlorophyll-*a*/L for *Fragilariopsis pseudonana*) in quadruplicate (12 bottles per species) and sealed with Teflon tape. For each pCO_2 level, triplicate blank bottles (3 bottles each pCO_2 treatment) containing no diatoms were included to ensure pH and DIC changes were attributed solely to the diatoms, and not from outgassing of the system. Each day, all bottles were inverted to ensure homogenous pCO_2 concentrations within the bottles and were subsequently randomly placed back in the incubator. After six days, the experiment was terminated. At termination, samples were filtered (10 mL) for chlorophyll measurement, and the remaining for particulate PUA analysis. After filtration, all filters were frozen until processing.

Carbonate Chemistry

Prior to inoculation with diatoms, the carbonate chemistry of the media was assessed using a ThermoScientific Orion Star A121 pH probe, Apollo SciTech AS-C3 DIC Analyzer, and an Ocean Optics S-UV-VIS flame spectrophotometer. At the beginning of the experiment, one bottle from each treatment was randomly selected for daily carbonate chemistry monitoring.

Each day, the pH probe was used to monitor water pH. Every three days, carbonate chemistry was assessed using the DIC analyzer and UV-vis spectrophotometer. For pH analysis, water samples were warmed to 24.95°C and transferred to a 5 cm jacketed cuvette. The samples received two injections of 20mL m-cresol dye and absorbance was measured at 434, 578, and 730 nm wavelengths after each injection. For DIC analysis, samples were brought to room temperature, injected into the DIC analyzer with 10% phosphoric acid, and measured for total DIC. On the final day, pH and DIC analysis was performed on the bottles as described above, including one additional sealed bottle from each treatment.

All spectrophotometric and DIC analysis data from days zero, three, and six were input into CO2SYS to calculate pH (total scale), total DIC, and pCO_2 (μatm) (Pelletier et al. 2012). Temperature and salinity at time of sampling were measured using the ThermoScientific pH probe.

Chlorophyll-a Quantification

Because cells of *S. marinoi* and *F. pseudonana* are small and difficult to count, chlorophyll-*a* was used to standardize PUA production between the cultures and pCO_2 treatments. Uniformly mixed cultures (10 mL) were filtered onto GF/C filters, placed in test tubes containing 90% acetone (6 mL), and stored at -20°C to allow for chlorophyll extraction. The next day, samples were brought to room temperature in darkness and decanted into glass test tubes. Chlorophyll-*a* was quantified using a Trilogy fluorometer on acidification mode by measuring fluorescence before and after acidification with 1 M sulfuric acid (4 drops, Lorenzen 1967). Chlorophyll measurements were performed in quadruplicate at time zero and once for each culture at the end of the experiment. Diatom growth rates were calculated using time zero and time final chlorophyll-*a* concentrations assuming exponential growth.

Reagents

High grade hexane (>95%, J.T.Baker®) and methanol (>99.5%, Macron Fine Chemicals™) were used for PUA extraction. *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA, derivatization grade >99%, Alfa Aesar) was used for PUA derivatization. Benzaldehyde (1000 µg/mL, SPEX CertiPrep) was used as internal standard. Hexadecane-*d*34 (98 atom%, Sigma-Aldrich) was used as recovery standard. PUA standards of 2E,4E-heptadienal (>90%, TCI America), 2E,4E-octadienal (90%, Ambeed, Inc.), and 2E,4E-decadienal (>90%, TCI America) were used for instrument calibration with purities accounted for.

Particulate PUA Method

Cultures were brought to the Lemkau lab in the WWU Chemistry department for PUA analysis. Diatoms were isolated via vacuum filtration onto GF/C (1.2 µm) glass fiber filter. Vacuum was maintained under 500 mBar to prevent diatom cell wall disruption (Vidoudez et al. 2011). Filters were transferred into 4 mL vials containing 1 mL PFBHA (25 mM) in Tris/HCl (100 mM, pH 7.0) derivatization agent and 5 µL benzaldehyde internal standard (1 mM) and frozen at -20°C. Samples were thawed completely, and the freeze/thaw process was repeated twice more to ensure cell lysing. After the last thaw, the samples were kept at room temperature for one hour to allow the derivatization reactions of released PUAs to occur. After derivatization, the samples were frozen again. After removal from the freezer, methanol (0.5 mL) was added to each sample, and they were thawed completely. Samples were extracted with hexane (1 mL), acidified with sulfuric acid (6 drops, 1 M) and centrifuged at 1735 g for 2 minutes to break any emulsion created by cell disruption. The organic layer was pipetted off and transferred to a clean

4 mL vial. Samples were extracted twice more with 0.5 mL and 0.3 mL hexane, respectively. Hexane extracts were combined, anhydrous sodium sulfate added to remove any remaining water, and dried extracts transferred to a clean 4 mL vial. Samples were dried under nitrogen. Residues were brought up in hexane (95 μL) and transferred to a GC vial with hexadecane-*d*34 (5 μL, 0.226 mg/mL) as a recovery standard. After vortexing, samples were wrapped with Teflon tape and stored at -80°C until GC-MS analysis the next day.

Gas Chromatography Parameters

Samples were analyzed via gas chromatography with detection via mass spectrometer (GC-MS). The instrument consisted of a HP 6890 split/splitless gas chromatograph equipped with an Agilent 7683 autosampler and coupled to a HP 5973 quadrupole mass spectrometer. Separations were performed on a Agilent HP-5MS column (30 m, 0.250 mm internal diameter with 0.25 μm film thickness) programmed from 60°C (2 minute hold), ramped to 240°C at 8°C/min, then to 285°C at 15°C/min. Helium was used as the carrier gas at a constant rate of 1.5 mL/min. Data was collected in SIM mode (100 ms dwell time). PUA identification was performed using their respective molecular ions, including m/z 305 (heptadienal), 319 (octadienal), and 347 (decadienal)- and of major ions- m/z 57 (alkanes), 66 (hexadecane-*d*34), 181 (all PFBHA-derivatized aldehydes), and 276 (all PUA molecules) (Table 1, Figure A2).

PUA Quantification

PUA quantification was performed using methods adapted from Vidoudez et al. (2011). In brief, PUAs were quantified by comparing the benzaldehyde internal standard peak area to each PUA molecular ion. Recoveries were calculated based on internal (benzaldehyde)

and external (hexadecane-*d*34) peak areas, taking into account instrument response factors. Response factors were calculated via an external calibration curve.

Method detection limit (MDL) and quantification limit (MQL) were determined following Glaser et al. (1981). These limits were used for PUAs produced by each culture, and PUAs above the limit of quantification were standardized using measured chlorophyll-*a* values (nmol per μg chlorophyll-*a*). Pre-standardization values below the quantification limit were substituted with MDL/2 for statistical analysis. MDL and MQL values are shown in Table 2.

Statistical Analyses

Two-way ANOVAs were used to assess for differences of PUA production between treatments. Prior to each analysis, the assumptions of normality and equal variance were tested using a Shapiro-Wilk test and Levene's test, respectively, using $\alpha = 0.05$. Log and square root transformations were performed on the data to meet the assumptions if needed, and tests were run using the transformed data. Tests included 1) production of individual PUA molecules across pCO_2 level in *S. marinoi*, 2) production of individual PUA molecules across pCO_2 level in *F. pseudonana*, and 3) production of total PUA molecules across pCO_2 level in both species. If significance was observed, Tukey's *post-hoc* tests were performed to assess differences across factor levels.

Results

Benthic Survey

All eight benthic diatom species surveyed produced at least one of the three monitored PUAs in detectable amounts (Figure 5). While production of at least one PUA was quantifiable for each diatom species, the amount of PUA produced varied between species, with means ranging from 0.6 pmol/µg chlorophyll-*a* (*L. communis*, decadienal) to 0.14 nmol/µg chlorophyll-*a* (*F. pseudonana*, octadienal). Both heptadienal and decadienal were detected in all eight species tested, while octadienal was detected in seven of the eight species. Replicate cultures in several diatom species had undetectable decadienal, thus data for these cultures is only based on one replicate.

Upwelling Experiment

Water Chemistry and Diatom Growth

Throughout the experiment, water chemistry in cultures containing diatoms decreased in pCO_2 concentration and increased in pH. pCO_2 levels in the pre-equilibrated media were slightly higher (4-6%) than target values of 400, 800, and 1200 µatm (Figure 6). In *S. marinoi* cultures, pCO_2 concentrations decreased, with greater than 90% uptake from initial pCO_2 concentrations. pH increased by approximately 1 pH unit for each culture (Figure 7). In *F. pseudonana* cultures, similar trends were observed. pCO_2 decreased by 75-81% and pH increased by about 0.5 pH units for each culture. No change in pH or decline in pCO_2 was observed in control bottles.

Between the two species and across all pCO_2 treatments, *S. marinoi* drew down more pCO_2 over the course of the experiment than *F. pseudonana* (Figure 6). *S. marinoi* consumed about 66, 131, and 193 µatm pCO_2 per day in the 400, 800, and 1200 µatm treatments,

respectively. *F. pseudonana* consumed about 53, 112, and 170 µatm pCO_2 per day at the same levels. Both species changed their carbon uptake rate depending on the pCO_2 treatment, drawing down more pCO_2 as pCO_2 concentration increased. This increase of carbon uptake cannot be attributed to a difference in biomass for either species. All bottles for each species received the same amount of inoculation biomass at the start of the experiment, and diatom growth rates were similar (Table 3).

PUA Results

For *S. marinoi*, pCO_2 level had no significant effect on the production of PUA molecules ($p = 0.294$, Figure 8a, b, and c). There was, however, a significant difference in the amount of the different PUA molecules (e.g., heptadienal produced at higher concentrations than decadienal), but this was independent of pCO_2 level (Figure 8a, b, and c, Table A5). No significant interaction was found between PUA molecule production and pCO_2 level ($p = 0.903$).

For *F. pseudonana*, a significant difference in PUA production was observed across pCO_2 level ($p = 0.026$, Figure 9a, b, and c). *Post-hoc* analysis indicated significantly higher production of PUAs in the 400 µatm pCO_2 treatment compared to 1200 µatm ($p = 0.028$). Similar to *S. marinoi*, significant differences in the amount of the PUA molecules produced by *F. pseudonana* were observed (e.g., octadienal produced at higher concentrations than decadienal), independent of pCO_2 level (Figure 9a, b, and c, Table A5). No significant interaction was found between PUA molecule and pCO_2 level ($p = 0.594$).

Comparing total PUA molecule production in both diatom species, *S. marinoi* produced significantly higher concentrations of PUAs than *F. pseudonana*, ranging from five- to nine-times higher depending on pCO_2 level ($p << 0.001$, Figure 8d, 9d).

Discussion

Major Findings

This is the first study to show PUA production in benthic diatoms outside of the Adriatic Sea. The results of the Salish Sea benthic PUA producer survey showed that all eight benthic diatom species isolated were found to be producers of at least two types of PUAs (Figure 5). Further, there was no evidence that PUAs serve as a carbon repository under elevated dissolved inorganic carbon concentration. In the upwelling experiment, only one treatment showed elevated pCO_2 levels had any effect on the production of PUA molecules in both diatom species but did impact carbon uptake rates in each species tested.

Benthic PUA Production in the Salish Sea

Given the ubiquity of PUA-producing diatoms in the pelagic zone and benthic presence in the Adriatic Sea, PUA-producing benthic diatoms were likely to be present in the Salish Sea. This was correct, as all eight benthic diatom species isolated produced PUA molecules in varying concentrations, depending on diatom species and molecule type. This result agrees with the Adriatic Sea benthic survey, where all three diatoms studied were found to produce some type of PUA (Pezzolesi et al. 2017). Most benthic diatoms surveyed here produced all three of the monitored PUAs, with octadienal produced in the highest concentrations and decadienal in the lowest. This result contradicts other PUA survey studies, including from pelagic diatoms, where heptadienal and other C7 PUAs were produced in the highest concentrations (Vidoudez et al. 2011). Decadienal and other C10 PUAs have consistently been observed in lower concentrations over C6, C7, and C8 PUAs (Wichard et al. 2005, Pezzolesi et al. 2017).

The disparity in concentrations of different PUAs produced by the benthic diatoms surveyed here prompts the question: why are PUA molecules produced in different quantities? Some studies exploring the fecundity reduction effect of PUAs on benthic invertebrates have found a difference in bioactivity of PUA molecules, with decadienal having the highest bioactivity and octadienal the lowest (Varella et al. 2014). This bioactivity was assessed, however, using individual PUA molecules. PUA molecules in mixtures have synergistic effects and are more toxic than individual PUAs alone; PUA mixtures can deform benthic invertebrate offspring at lower concentrations in comparison to single PUAs (Romano et al. 2010, Ruocco et al. 2019). Given this difference in bioactivity and mixture synergy, diatoms may contain varying amounts of different PUA precursor molecules to cause an embryogenic effect at lower concentrations.

Given PUA's documented impact on predator fecundity, widespread production of PUAs in benthic diatoms of the Salish Sea has significant ecological implications (Romano et al. 2010, Varella et al. 2014, Ruocco et al. 2019). Benthic diatoms are the primary food source for many benthic consumers, including protists, molluscs, and other invertebrates like crabs (Fenchel 1968, Zimba et al. 2016, Fenoglio et al. 2020, Haynert et al. 2020). In addition, benthic diatoms bloom in early spring, when benthic invertebrates (e.g., intertidal snails) reproduce (Spight 1976). The synchrony of these events suggests that benthic diatoms could be a primary food source during invertebrate reproduction, and diatom PUAs a pathway for disrupted embryogenesis in invertebrate offspring (Varella et al. 2014). When organisms consume PUA-producing diatoms, inadvertent accumulation of PUAs in gonadal tissues can cause a direct transfer of PUAs into offspring eggs where lipid requirement is high, which may reduce egg viability, hatching success, and embryogenesis, as is seen in copepods (Ban et al. 1997, Ianora et

al. 1999). Benthic diatom consumers link primary production to higher trophic levels of the pelagic and benthic environments. If benthic PUA production causes a negative effect on consumer fecundity, then widespread PUA production could mean that benthic-derived PUAs have a greater influence on marine food webs than previously thought. In addition, various benthic diatoms, including the diatom *C. closterium* (found to be a PUA producer in the current study), have recently been used and recommended as a food source for population recovery of an endangered species of abalone, *Haliotis kamtschatkana* (Kuehl 2020). While benthic diatom diets may support organismal somatic growth and development, long-term feeding may have fecundity repercussions that delay overall recovery.

Upwelling Experiment

While elevated pCO_2 could reasonably be thought to increase PUA concentrations within diatom cells due to more efficient carbon uptake, I found that pCO_2 enrichment had little effect on PUA dynamics. In *S. marinoi*, no significant difference was observed in PUA production across pCO_2 level. However, a minor effect was observed in *F. pseudonana*, whereby lower PUA production was observed at 1200 µatm pCO_2 than at 400 µatm pCO_2.

The pCO_2 concentrations that diatoms were exposed to impacted their carbon uptake rates. Both diatoms increased their pCO_2 uptake rate under higher initial pCO_2 concentrations, a finding consistent with other carbon enrichment studies (Taucher et al. 2015, Bach et al. 2019). Given that diatom growth rates were the same across pCO_2 level, this suggests that carbon is being allocated to something other than population growth. In addition, an increase in environmental pCO_2 can cause a down-regulation of CCMs, as pCO_2 is acquired more efficiently from the environment at higher concentrations (Wu et al. 2010, Shi et al. 2015). If CCMs are

downregulated, both more energy and carbon are available to the diatoms, which could lead to greater production of secondary metabolites, such as PUAs. While the diatoms drew down increasing levels of pCO_2, my findings showed little effect of pCO_2 on PUA production. This suggests that carbon-based molecules other than PUAs are produced. In a mesocosm study exploring the effect of elevated pCO_2 on plankton communities, Riebesell et al. (2007) found a four-fold increase in concentration of carbon-rich transparent exopolymer particles under elevated pCO_2 conditions. In the current work, *F. pseudonana* PUA production was significantly lower in the 1200 µatm treatment (Figure 9). Excess carbon uptake in this CO_2-enriched treatment could have gone towards production of exopolymers and other metabolites. This suggests pathways beyond PUA production for organically fixed carbon in high pCO_2 concentrations, and excess production of these metabolites is a topic for future studies.

Diatom grazers like copepods are the link between primary production and higher trophic levels. If elevated pCO_2 level and a resultant increase in diatom PUAs was observed, any reduced grazer fecundity from elevated PUAs would have potential cascading ecological effects. For example, a decline in copepod and/or invertebrate grazer offspring through PUA ingestion would cause a multi-level trophic effect in an ecosystem, whereby future secondary production would be inhibited. However, the results here indicate that elevated levels of pCO_2 did not increase PUA production in diatoms, and therefore seasonal upwelling is likely not causing an increase in PUA production. If increased exopolymer production results from diatom exposure to elevated DIC, there may be an impact on the efficiency of the biological pump (Sigman & Hain 2012, Basu & Mackey 2018). Instead of increased DIC uptake leading to particulate diatom production, the inorganic carbon is seemingly fixed to dissolved forms of organic carbon, thus fueling the microbial loop rather than fueling high export production (Daly et al. 2021). In

contrast, an increase in exopolymer production could cause greater amounts of marine snow aggregation, increasing export and therefore efficiency of the biological pump (Turner 2015).

Not explored in this study is the potential synergy between pCO_2 level and nutrient limitation. PUA production is impacted by nutrient availability, particularly nitrogen and phosphorus, likely because of their impact on protein and enzyme formation (Ribalet et al. 2009, Vidoudez 2011, Cózar et al. 2018). Lipoxygenase enzymes are needed to process PUA-precursors and form PUA molecules, and limiting protein-building nutrients would impact enzyme expression (Orefice et al. 2022). Whereas upwelling delivers elevated pCO_2 concentrations and nutrients to surface waters seasonally, oceanic pCO_2 concentrations are expected to rise as climate change intensifies without the seasonal delivery of inorganic nutrients (Gallego et al. 2018). Future studies could assess PUA production under ocean acidification scenarios (e.g., elevated pCO_2 and limiting nutrients) to test this synergy. This could reveal PUA production dynamics tied to pCO_2 levels not explored in the current work and may help us understand future diatom trophic dynamics.

Future Directions

The results of the benthic diatom survey highlight the need to include benthic diatoms in PUA research. Most studies exploring the effects of PUAs on benthic invertebrates have only used dissolved PUAs to assess their impact on predator fecundity, not direct uptake of particulate PUAs via maternal diatom consumption (e.g., Varella et al. 2014). Predator fecundity studies using PUA-producing pelagic diatom diets have shown similar responses to particulate and dissolved PUAs (Ianora et al. 1999, Miralto et al. 1999). The confirmation of benthic PUA producers in the Salish Sea highlights this gap in research. In future studies, benthic invertebrates

that consume diatoms can be fed PUA producers to replicate *in situ* trophic dynamics using particulate PUAs, and fecundity assessed.

Caldwell et al. (2004) have also shown that benthic invertebrate sperm density and motility decreased when exposed to dissolved PUAs. Given many benthic invertebrates utilize broadcast spawning for reproduction, the presence of dissolved PUAs could have a broader impact on trophic dynamics than previously thought (Crimaldi & Zimmer 2014). Benthic diatoms also inhabit substrates like eelgrass, where organisms like forage fish and invertebrates lay their eggs (Ruocco et al. 2019). Thus, organisms and their offspring are in the immediate environment of PUA-producing diatoms through numerous stages of faunal reproduction and development. Dissolved PUA concentrations are yet to be measured in the Salish Sea, and their measurement and seasonal monitoring could be useful in assessing their potential role on other trophic dynamics in the Salish Sea.

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Figures and Tables

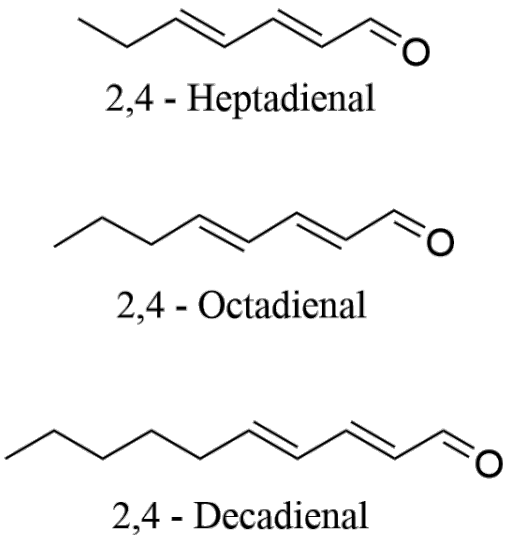


Figure 1. Chemical structures of the three PUAs considered in this study.

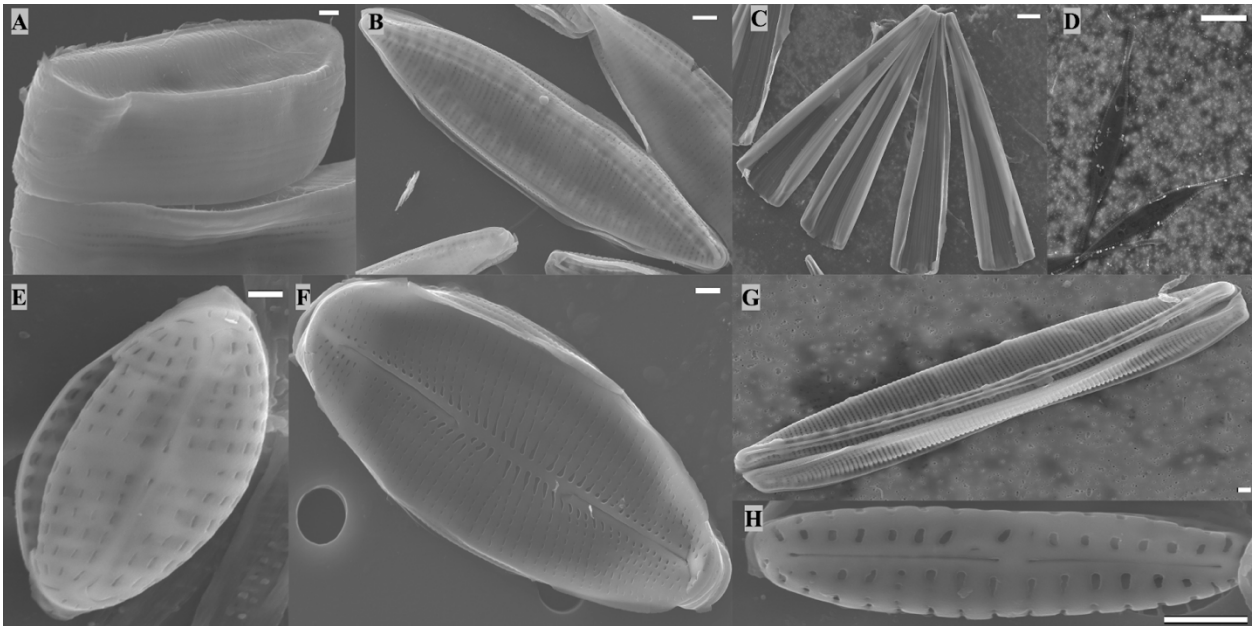


Figure 2. Representative scanning electron micrographs used for identification of (A) *Fragilariopsis pseudonana,* (B) *Nitzschia* sp. a, (C) *Licmophora flabellata*, (D) *Cylindrotheca closterium*, (E) *Navicula vara*, (F) *Fistulifera saprophila*, (G) *Nitzschia* sp. b, and (H) *Navicula* sp. White scale bars depict 1 μm in all images but (C) and (D), which depict 10 μm.

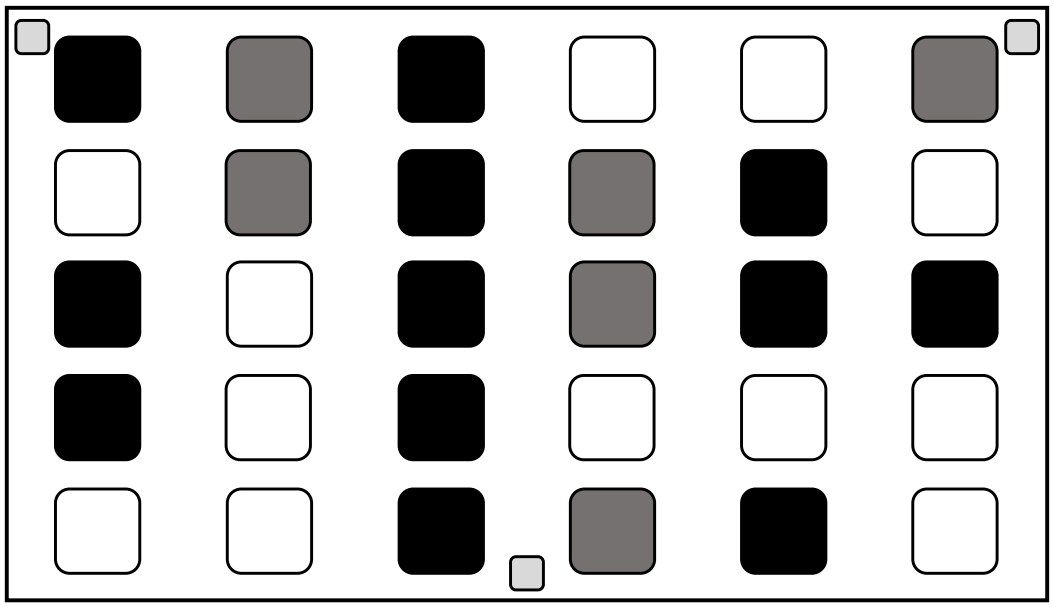


Figure 3. Representative example of culture distribution within the incubator during the upwelling experiment. Colors denote *S. marinoi* (white), *F. pseudonana* (black), and blanks (dark gray). Temperature loggers (light gray) were placed in corners of the incubator. Culture placement was randomized daily.

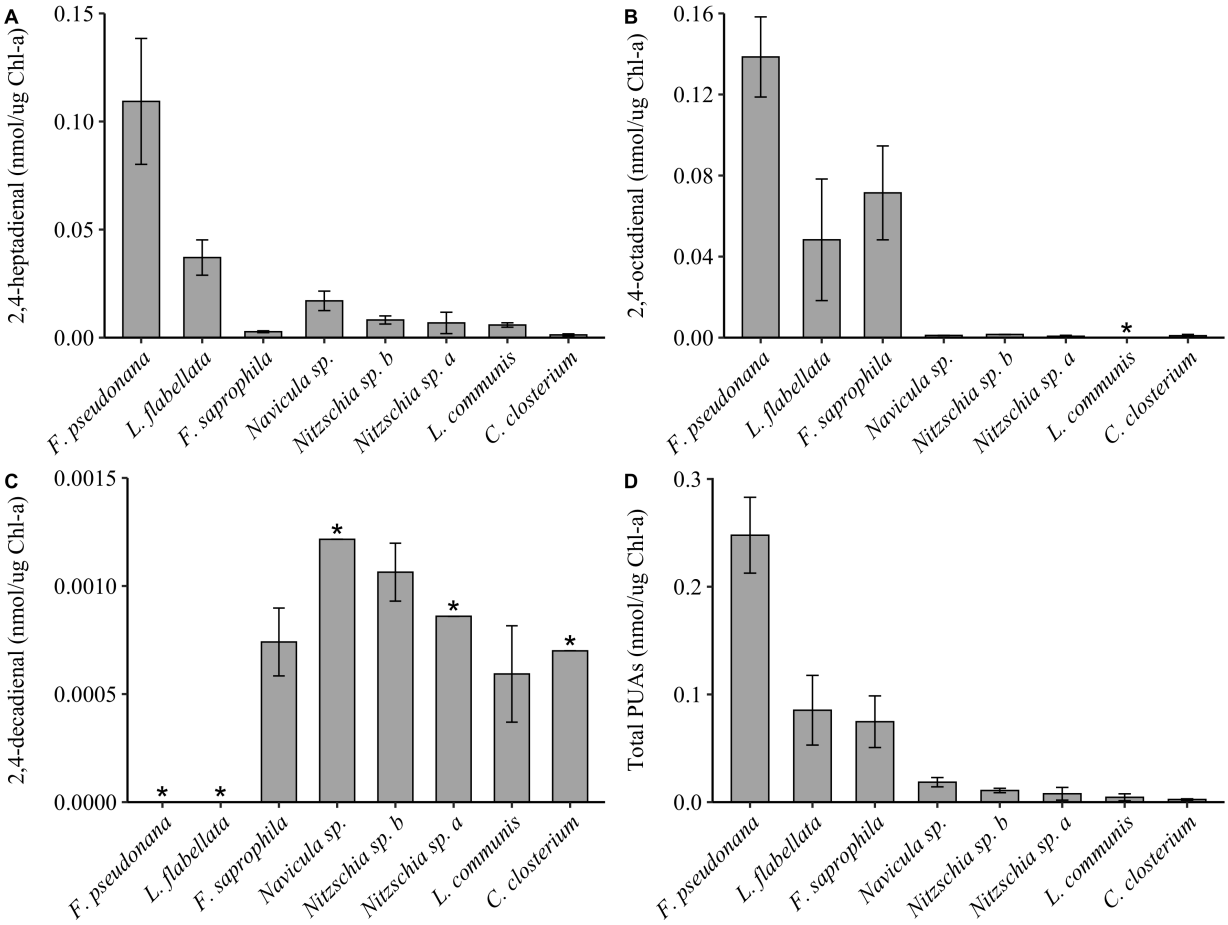


Figure 4. Benthic diatom PUA survey of (A) 2,4-heptadienal, (B) 2,4-octadienal, (C) 2,4-decadienal, and (D) total PUA production. Gray bars represent means of quantifiable replicates and error bars represent standard deviations. Each diatom species tested was performed in triplicate cultures and quantified as nmol PUA molecule per µg of chlorophyll-*a*. Cultures containing replicates below method quantitation limit (MQL) are marked with an asterisk. Note differences in scale of Y axes.

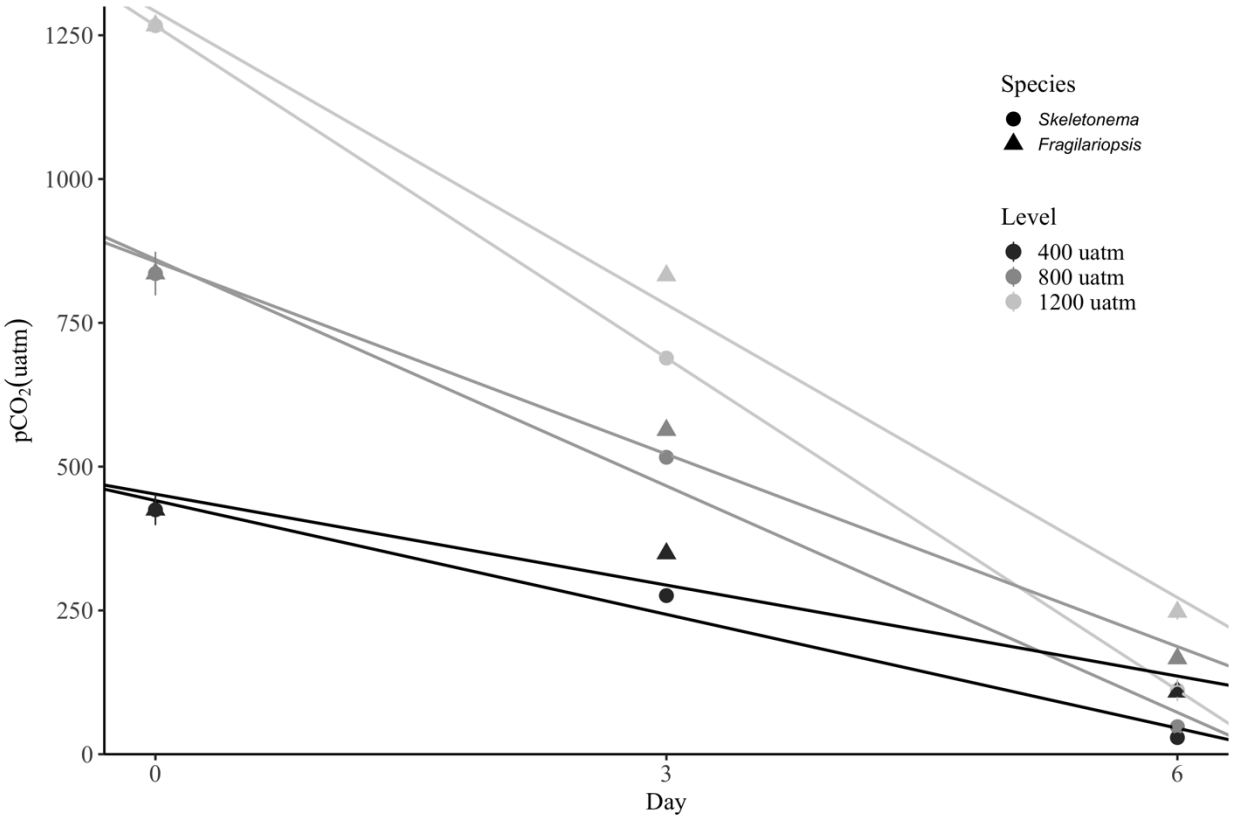


Figure 5. Carbon acquisition plot of diatoms throughout the upwelling experiment. Both species started in media pre-equilibrated to target pCO_2 levels (µatm). Symbol shape is used to define the diatom species, while color is used to define pCO_2 level. Trendlines are used to infer daily CO_2 acquisition rate. Pre-equilibrated media (Day 0) and Day 6 values are means of quadruplicate and triplicate measurements, respectively. Error bars represent standard deviations, with some bars are hidden behind symbol. Day 3 values come from single measurements.

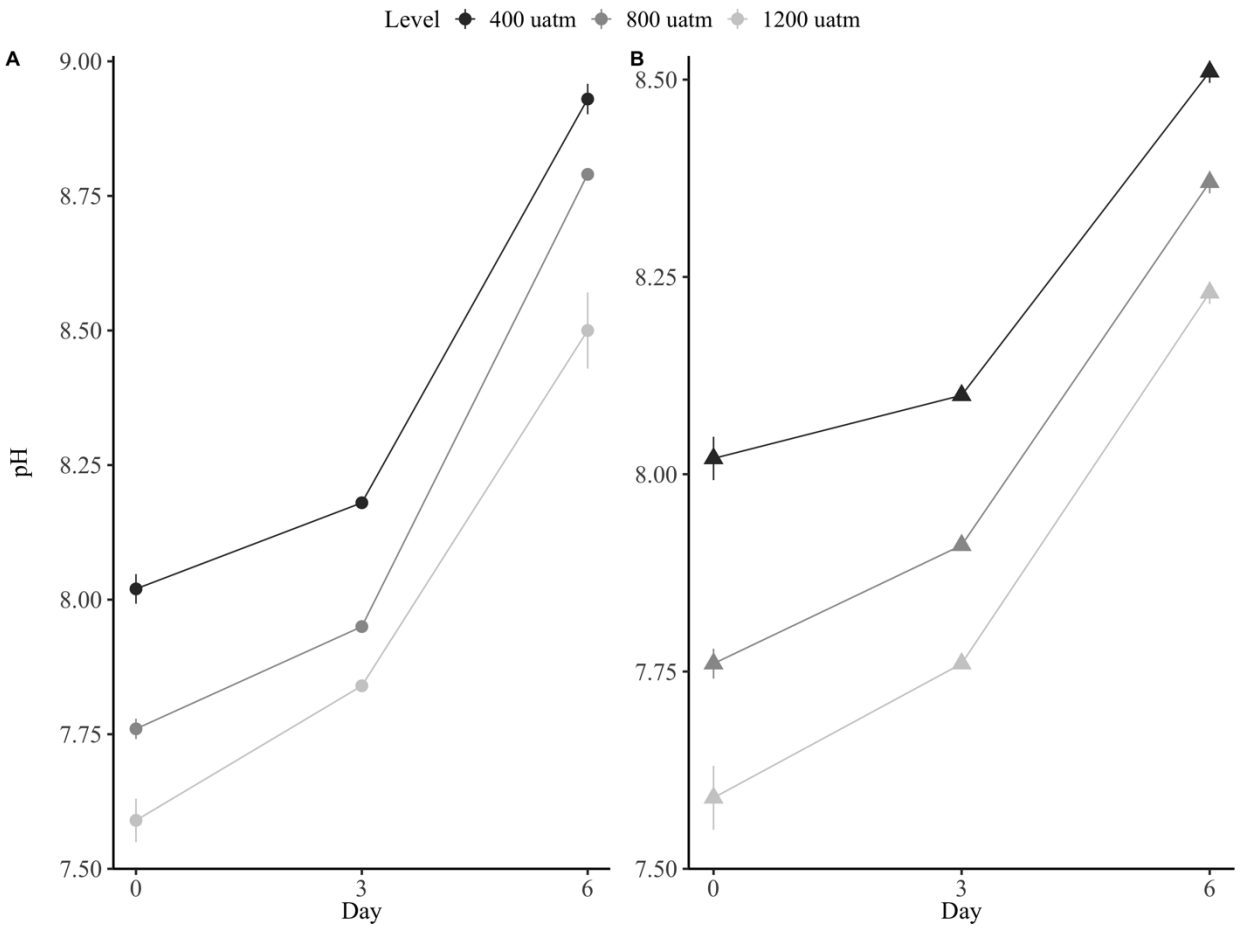


Figure 6. pH measurements of media from (A) *S. marinoi* and (B) *F. pseudonana* throughout the upwelling experiment. Color is used to define pCO_2 level (µatm). Pre-equilibrated media (Day 0) and Day 6 values are means of quadruplicate and triplicate measurements, respectively. Error bars represent standard deviations. Day 3 values come from single measurements. Note the difference in Y axes scale.

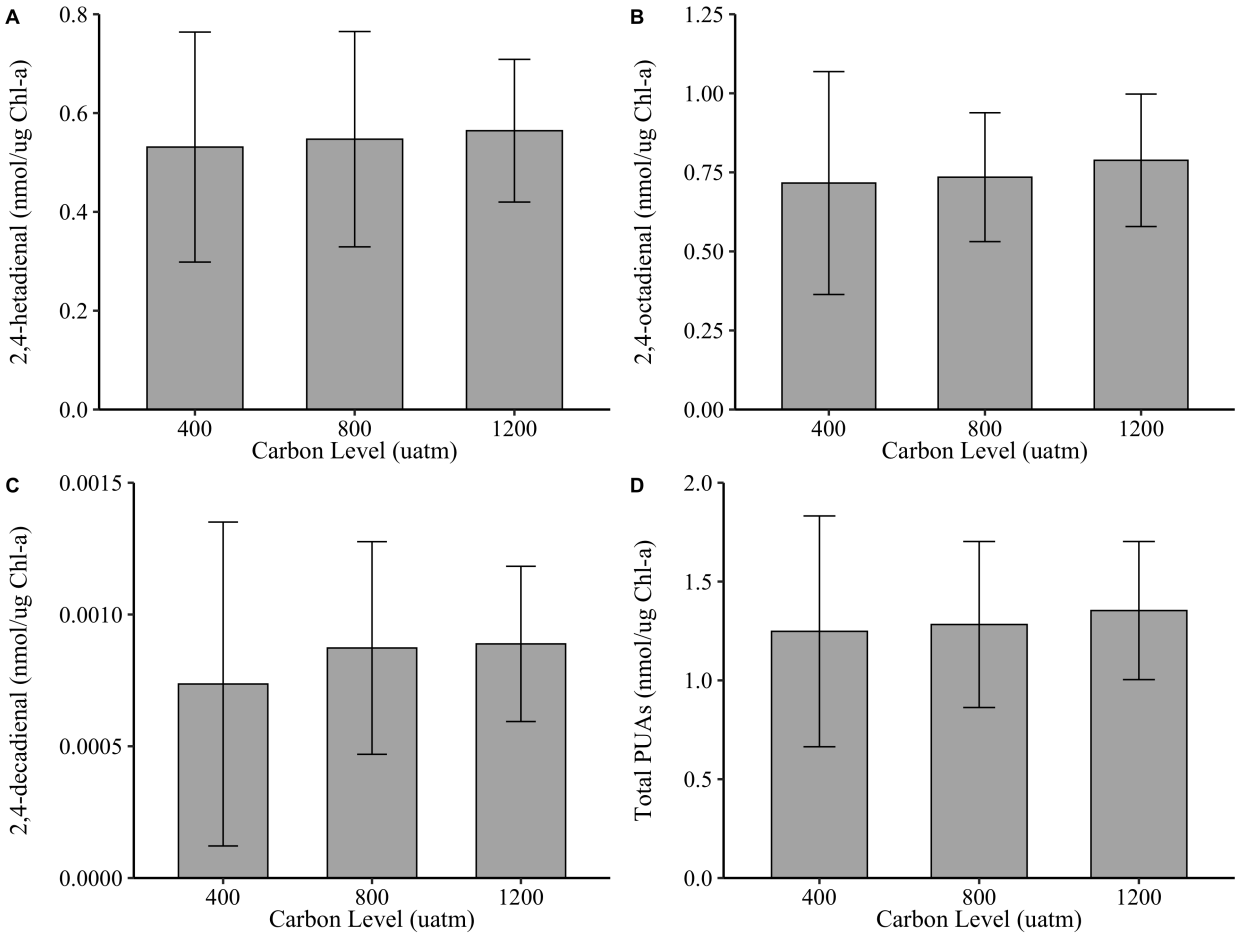


Figure 7. *S. marinoi* PUA production across pCO_2 level (μatm) of (A) 2,4-heptadienal, (B) 2,4-octadienal, (C) 2,4-decadienal, and (D) total PUA production. Gray bars represent means of replicate cultures and error bars represent 95% confidence intervals. No significant difference was observed across pCO_2 level ($p = 0.294$). *S. marinoi* produced greater concentrations of 2,4-octadienal than 2,4-heptadienal ($p = 0.005$) and lower amounts 2,4-decadienal ($p << 0.001$) across all pCO_2 levels. Note differences in scale of Y axes.

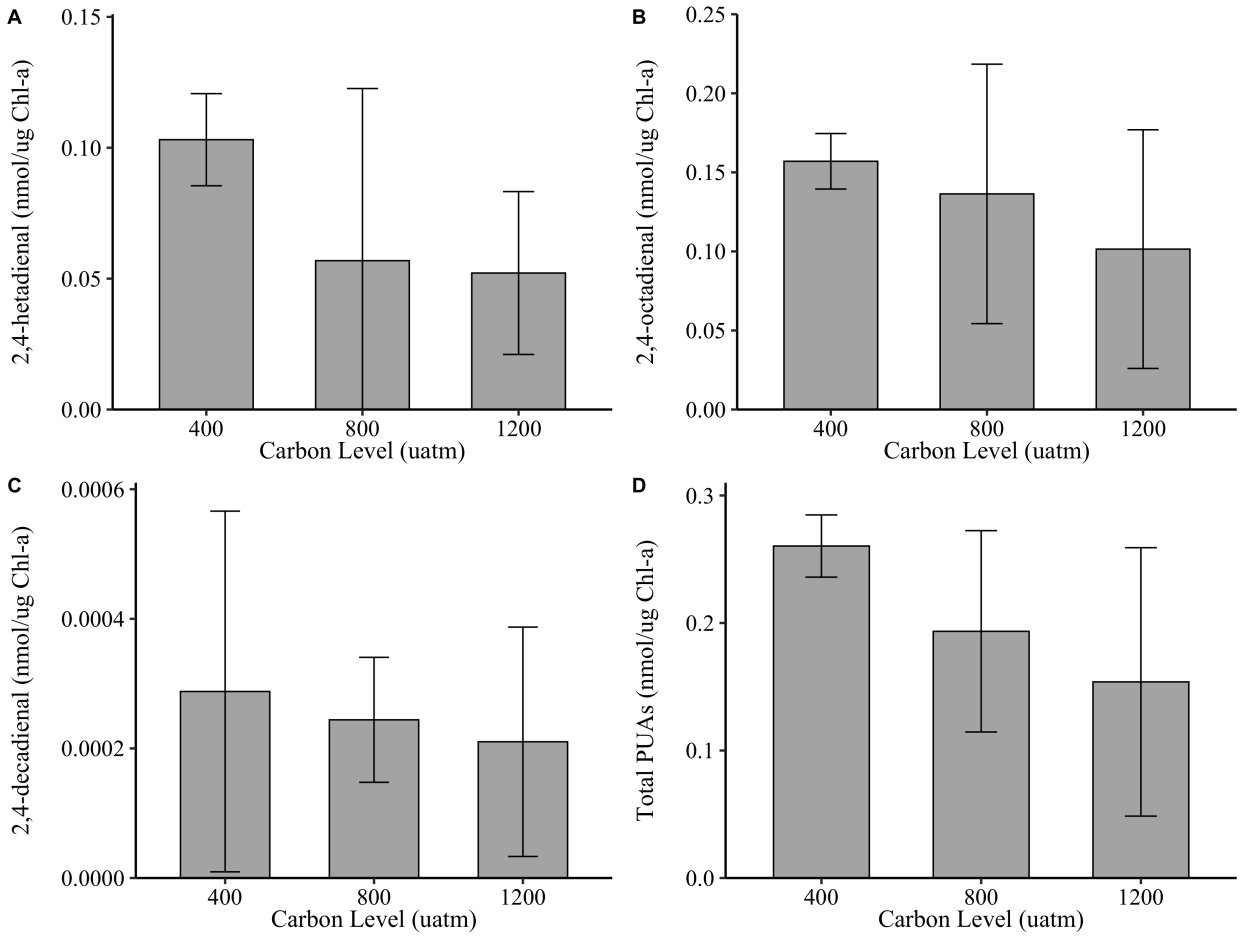


Figure 8. *F. pseudonana* PUA production across pCO_2 level (µatm) of (A) 2,4-heptadienal, (B) 2,4-octadienal, (C) 2,4-decadienal, and (D) total PUA production. Gray bars represent means of quantifiable replicate cultures and error bars represent 95% confidence intervals. *F. pseudonana* produced significantly more PUAs in the 400 µatm treatment than the 1200 µatm treatment ($p = 0.028$). *F. pseudonana* also produced greater concentrations of 2,4-octadienal than 2,4-heptadienal ($p = 0.005$) and lower amounts 2,4-decadienal ($p << 0.001$) across all pCO_2 levels. Values below the quantification limit were quantified and visualized using MQL/2. Note differences in scale of Y axes.

Table 1. Molecular and fragment ions (*m/z*) of compounds monitored for identification on GC-MS.

Compound	Molecular Ion (*m/z*)
Alkanes	57
Hexadecane-*d*34	66
PFBHA Derivatization Agent	181
All PUAs	276
Benzaldehyde	301
2,4-Heptadienal	305
2,4-Octadienal	319
2,4-Decadienal	347

Table 2. Method detection limit (MDL) and quantitation limit (MQL) of PUA molecules. Values were calculated using 6 replicate 0.001 nmol mixed PUA standards. MDL was calculated as 3σ and MQL as 10σ.

Compound	MDL (nmol)	MQL (nmol)
2,4-Heptadienal	0.00556	0.0185
2,4-Octadienal	0.00526	0.0175
2,4-Decadienal	0.00858	0.0286

Table 3. Average chlorophyll-*a* measurements (µg) of inoculum and experimental bottles with their respective growth rates (µ). Errors are standard deviations based on four replicate analyses. Growth rates were calculated using pre- and post-experiment chlorophyll measurements assuming exponential growth. No significant difference was found in growth rates and total chlorophyll-*a* across pCO_2 levels within species.

Sample	**Total *S. marinoi* chlorophyll-*a* (µg)**	***S. marinoi* Average µ**	**Total *F. pseudonana* chlorophyll-*a* (µg)**	***F. pseudonana* Average µ**
Inoculum	0.87 ± 0.17	-----	1.99 ± 0.09	-----
400 µatm	98.02 ± 6.30	0.79 ± 0.01	66.43 ± 6.55	0.58 ± 0.02
800 µatm	105.42 ± 21.48	0.80 ± 0.03	72.78 ± 17.14	0.60 ± 0.04
1200 µatm	96.19 ± 11.17	0.78 ± 0.02	80.03 ± 38.13	0.60 ± 0.08

Appendix A

Table A1. Water chemistry parameters for pre-equilibrated media and diatom cultures. Variables include salinity (ppt), pH, DIC (µmol/kg), and pCO_2 (µatm). Day 0 media and Day 6 values are means ± standard deviations of quadruplicate and triplicate measurements, respectively.

pCO_2 Level (µatm)	Salinity (ppt)	pH	DIC (µmol/kg)	pCO_2 (µatm)
Pre-Equilibrated Media				
400	32.13	8.02 ± 0.03	2034 ± 1	425 ± 27
800	32.15	7.76 ± 0.02	2141 ± 2	836 ± 38
1200	32.21	7.59 ± 0.04	2191 ± 3	1267 ± 110
S. marinoi				
Day 3				
400	32.38	8.18	1980	276
800	32.40	7.95	2094	516
1200	32.46	7.84	2143	689
Day 6				
400	32.30 ± 0.04	8.93 ± 0.03	1619 ± 0	29 ± 3
800	32.33 ± 0.01	8.79 ± 0.00	1777 ± 6	48 ± 0
1200	32.36 ± 0.04	8.50 ± 0.06	1802 ± 2	112 ± 19
F. pseudonana				
Day 3				
400	32.37	8.10	2009	349
800	32.33	7.91	2068	563
1200	32.39	7.76	2116	832
Day 6				
400	32.38 ± 0.13	8.51 ± 0.02	1822 ± 4	108 ± 5
800	32.36 ± 0.04	8.37 ± 0.01	1910 ± 9	166 ± 6
1200	32.45 ± 0.11	8.23 ± 0.02	1990 ± 9	247 ± 14

Table A2. Levene's analysis output for diatom PUA production across all PUA molecules and pCO_2 with degrees of freedom (df), F-values, and p-values. Assumption of equal variance is met where $p > 0.05$.

Comparison	df	F-value	p-value
S. marinoi			
pCO_2 – PUA Molecule	8	0.6511	0.7284
F. pseudonana			
pCO_2 – PUA Molecule	8	1.2478	0.3107
Total PUAs			
pCO_2 - Species	5	1.8217	0.1593

Table A3. Shapiro-Wilks analysis output for diatom PUA production across all PUA molecules and pCO_2 with p-values. Assumption of normality is met where $p > 0.05$. One analysis did not meet the assumption, which was acknowledged but ignored because ANOVA is robust to non-normality.

Comparison	p-value
S. marinoi	
pCO_2 – PUA Molecule	0.3348
F. pseudonana	
pCO_2 – PUA Molecule	0.0096
Total PUAs	
pCO_2 - Species	0.5062

Table A4. Two-way ANOVA output for diatom PUA production across all PUA molecules, pCO_2 levels, and species. Analysis was significant where $p < 0.05$.

Analysis	df	F-value	p-value
S. marinoi			
pCO_2	2	1.281	0.294
PUA Molecule	2	3600	<< 0.001*
F. pseudonana			
pCO_2	2	4.259	0.0247*
PUA Molecule	2	727	<< 0.001*
Total PUAs			
pCO_2	2	0.441	0.650
Species	1	441	<< 0.001*

Table A5. Tukey's *post-hoc* analysis output for diatom PUA molecules and pCO_2 levels. Analysis was significant were $p < 0.05$. Only significant results are shown.

Comparison	p-value
S. marinoi	
Heptadienal - Octadienal	0.0052
Heptadienal - Decadienal	<< 0.001
Octadienal - Decadienal	<< 0.001
F. pseudonana	
400 - 1200 µatm	0.024
Heptadienal - Octadienal	0.0013
Heptadienal - Decadienal	<< 0.001
Octadienal - Decadienal	<< 0.001

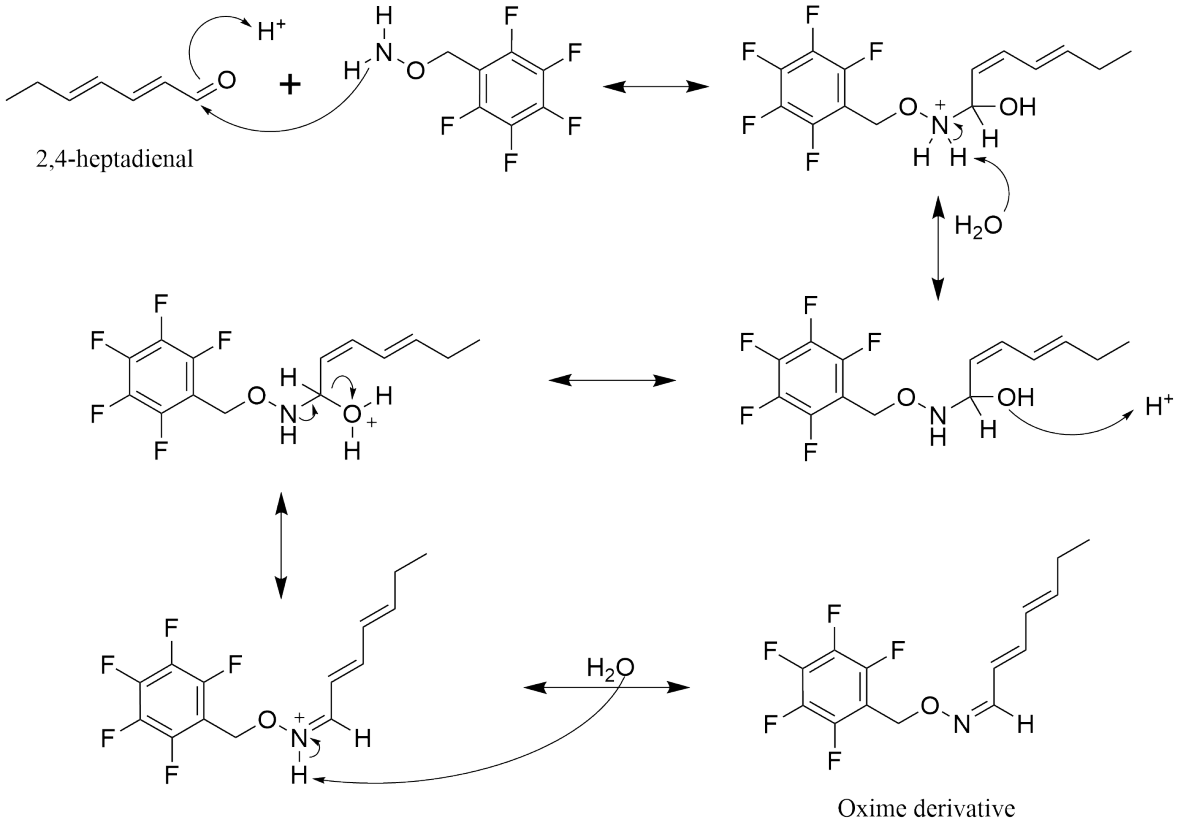
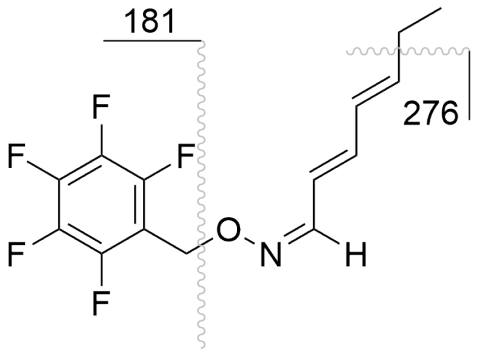


Figure A1. Reaction mechanism for derivatization of PUAs by PFHBA derivatization agent. Schematic shows reaction for 2,4-heptadienal as a representative example. Adapted from Klein (2011).



m/z = 305

Figure A2. Mass spectrometry fragmentation patterns used for identification of PFHBA derivatization agent fragment (*m/z* 181) and PUA molecules (*m/z* 276). Representative PUA 2,4-heptadienal (*m/z* 305) was used for the schematic.

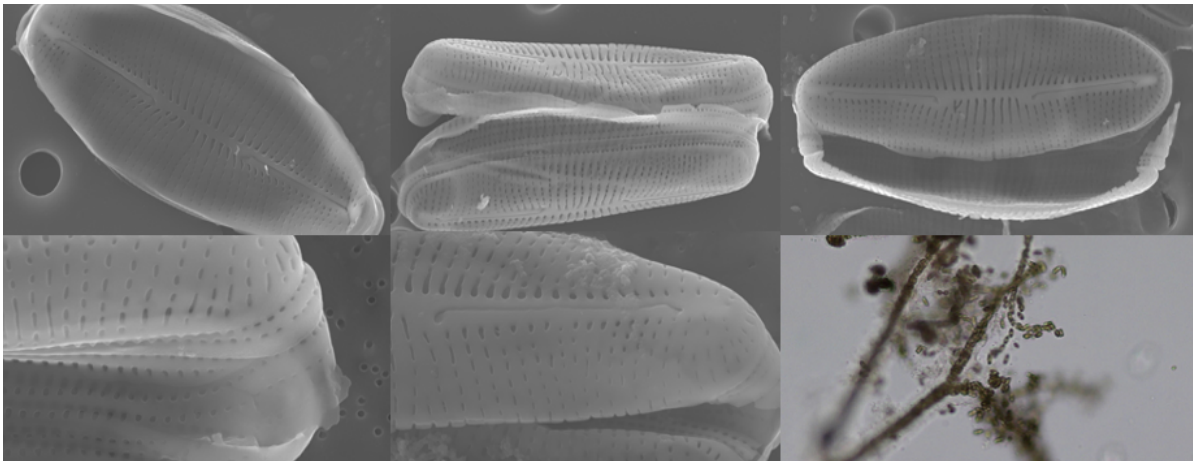


Figure A3. Scanning electron micrographs and light scope image used for identification of *Fistulifera saprophila*.

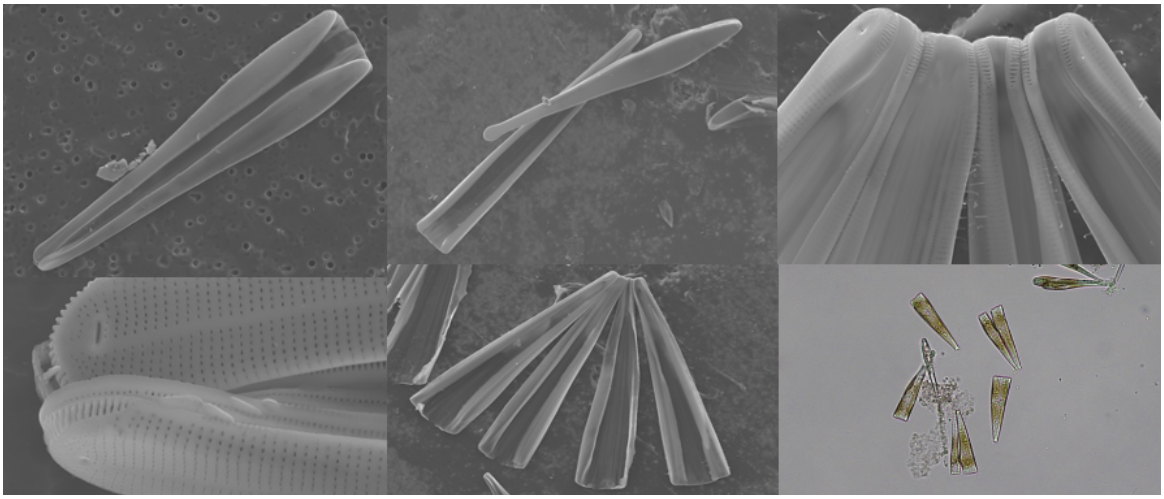


Figure A4. Scanning electron micrographs and light scope image used for identification of *Licmophora flabellata*.

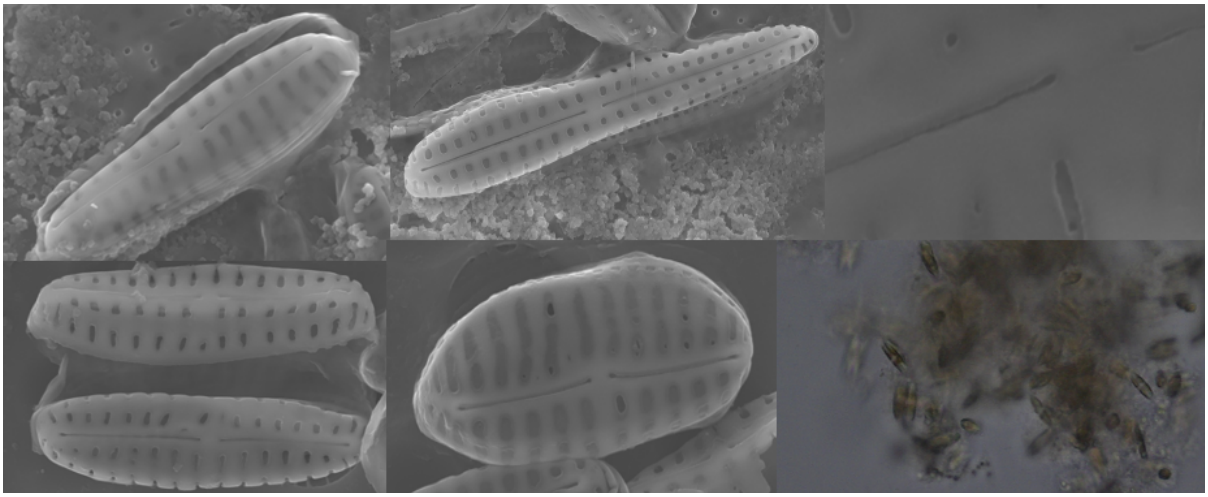


Figure A5. Scanning electron micrographs and light scope image used for identification of *Navicula* sp.

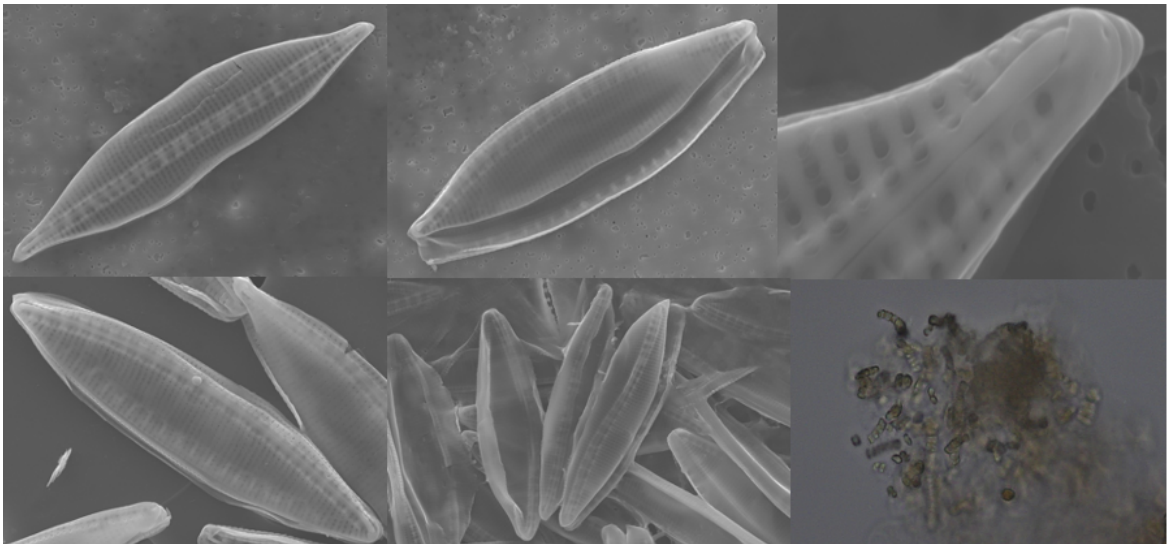


Figure A6. Scanning electron micrographs and light scope image used for identification of *Nitzschia* sp. a.

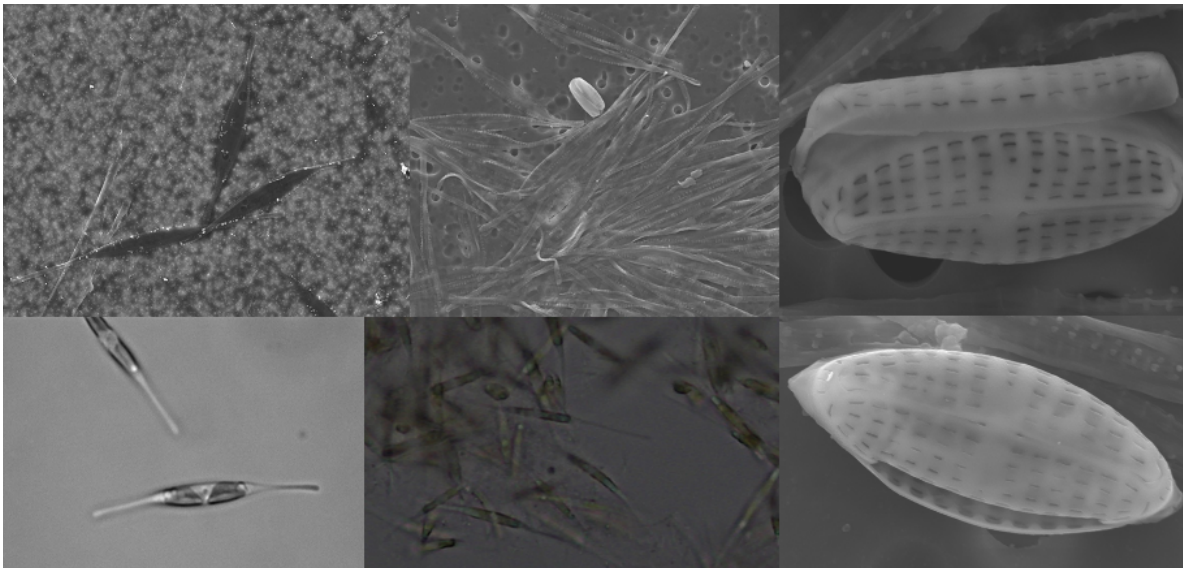


Figure A7. Scanning electron micrographs and light scope images used for identification of *Cylindrotheca closterium* and contaminant *Navicula vara*.

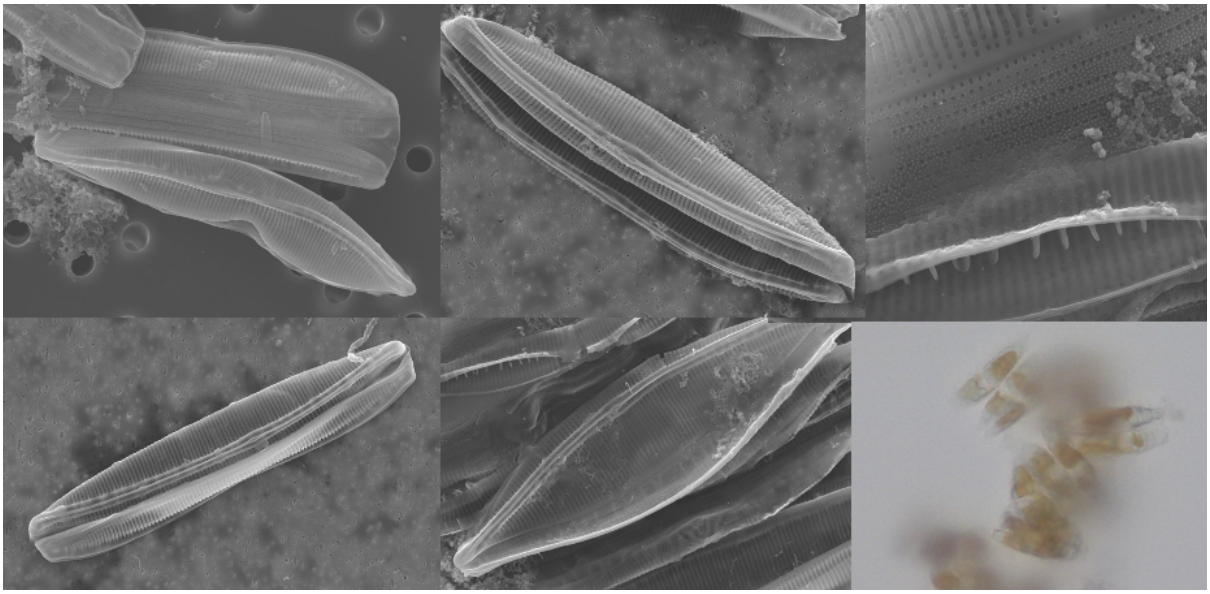


Figure A8. Scanning electron micrographs and light scope image used for identification of *Nitzschia* sp. b.

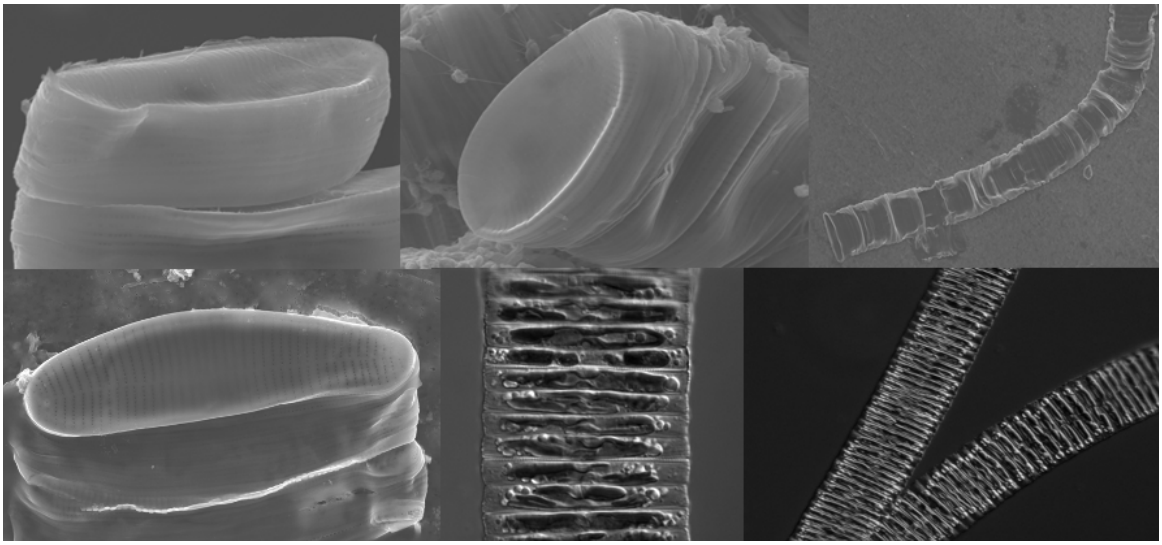


Figure A9. Scanning electron micrographs and light scope images used for identification of *Fragilariopsis pseudonana*.