Diatom-produced allelochemicals trigger trophic cascades in the planktonic food web

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Abstract

Diatoms produce a series of cytotoxic secondary metabolites such as polyunsaturated aldehydes (PUA) in response to cell injury or stress. However, little information exists on the PUA effects on the pelagic food web. A set of experiments was conducted in the Chesapeake Bay and the coastal Atlantic waters using dissolved PUA (2E,4E-octadienal and 2E,4E-heptadienal), natural assemblages of phytoplankton and microzooplankton, and the copepod Acartia tonsa. The results demonstrate that PUA primarily acts as deterrent for microzooplankton herbivory on diatoms, while enhancing herbivory on picophytoplankton. This switch should favor PUA-producing diatoms by simultaneously reducing direct grazing losses and competition. Additionally, PUA stimulated copepod predation on microzooplankton, particularly on ciliates. Combined, these effects have the potential to disrupt growth-grazing equilibrium in the pelagic food web and create an opportunity for bloom development.

Phytoplankton blooms occur at various scales in most regions of the ocean (e.g., Legendre 1990; McGillicuddy et al. 2007). Among them, diatom blooms have a great ecological and biogeochemical significance (Nelson et al. 1995; Smetacek 1999; Arrigo et al. 2012). Diatoms tend to dominate phytoplankton communities under nutrient-replete conditions such as in coastal waters and upwelling and frontal zones but also seasonally in the open ocean (Sarthou et al. 2005). Despite multi-decadal efforts, factors controlling plankton blooms remain controversial. Most bloom research has been focused on physical bottom-up factors controlling diatom cell division rate such as light, temperature, and dissolved minerals (e.g., Sverdrup 1953; Huisman et al. 1999). However, these factors do not universally explain blooms and decoupling between phytoplankton cell division and their biomass accumulation in the ocean (Behrenfeld 2010). An emergent alternative view emphasizes top-down factors (Bakun and Broad 2003; Irigoien et al. 2005). Specifically, it proposes that the primary reason for blooms is a temporal disruption of phytoplankton growth-herbivory equilibrium due to physical forcing such as deep winter mixing, freshwater input, upwelling or a food web shift (Behrenfeld and Boss 2014).

Traditionally, mesozooplankton (i.e., consumers between 200 μm and 2000 μm) such as planktonic copepods have been considered the dominant grazers of diatoms (Smetacek 1999). Copepods have more complicated development patterns and life histories than protistan microzooplankton (Litchman et al. 2013) that are incompatible with rapid response times and may allow phytoplankton to attain high biomass in the spring. Recent evidence shows that ciliates are an essential food source for copepods under most conditions, whereas diatoms contribute relatively little to their diet except in very productive systems during peak diatom biomass (Campbell et al. 2009; Saiz and Calbet 2011; Ray et al. 2016). Omnivory is common among pelagic copepods such as Acartia tonsa, which switches between diatom and ciliate prey (Kiørboe et al. 1996). The latter group of pelagic protists, together with phagotrophic dinoflagellates, form the bulk of microzooplankton (sensu stricto consumers between 20 μm and 200 μm) biomass, which is equal to that of mesozooplankton (Buitenhuis et al. 2010).

Microzooplankton herbivory is recognized as a major factor controlling primary production in the ocean (Calbet and Landry 2004). The ability of dinoflagellates (Hansen and Calado 1999; Olson and Strom 2002; Siano and Montresor 2005; Yoo et al. 2009) and ciliates (Hamels et al. 2004; Aberle et al. 2007; Sherr et al. 2013) to feed on diatoms, including large and chain-forming taxa is well documented. In addition, microzooplankton grazing can alter the structure of phytoplankton...
communities with ecosystem level effects. For example, microzooplankton were implicated in eliciting rapid change from single cells to multi-cell colonies in the prymnesiophyte *Phaeocystis* (Jakobsen and Tang 2002; Verity et al. 2007). In contrast to copepods, these grazers possess growth rates that match and even exceed those of their algal prey even at sub-zero temperatures (Franzè and Lavrentyev 2014 and references therein) and can respond rapidly to an increase in phytoplankton biomass (e.g., Levensen et al. 2000). Predation on microzooplankton by copepods dampens the primary herbivore response to phytoplankton biomass increase (Nejstgaard et al. 1997; Leising et al. 2005a; Löder et al. 2011). Such trophic cascades can play a pivotal role in controlling bloom dynamics (Vadstein et al. 2004, Flynn and Irigoien 2009; Armengol et al. 2017).

Typically, phytoplankton blooms are formed by a limited number of opportunistic species, which are not necessarily the best competitors for resources or the fastest dividers, but the least attractive for grazers due to various defenses (Jakobsen and Tang 2002; Hamm et al. 2003). Allelopathy (here production of chemicals by algae to inhibit the growth of competitors and deter grazers) is wide spread among phytoplankton (Strom 2008; Van Donik et al. 2011) and is thought to lead to bloom development in diatoms (e.g., Bakun and Broad 2003; Irigoien et al. 2005). Many diatom species produce a series of cytotoxic secondary metabolites collectively termed oxylipins, which result from the oxidative cleavage of polyunsaturated fatty acids when the cell is under stress or damaged (Ianora et al. 2012).

The polyunsaturated aldehydes (PUA) first identified in diatoms (Miralto et al. 1999) and subsequently in the prymnesiophyte *Phaeocystis pouchetii* (Hansen et al. 2004) are the best-studied group of phytoplankton-produced oxylipins. For example, octadecenal and heptadecenal production in the best-studied group of phytoplankton-produced oxylipins. For *Phaeocystis pouchetii* diatoms (Miralto et al. 1999) and subsequently in the prymnesiophyte *Skeletonema marinoi* is triggered upon cell disruption with a maximum yield of up to 9.8 fmol cell⁻¹ (Wichard et al. 2005; Ribai et al. 2007a). This species forms dense blooms in the northern Adriatic Sea (Sarno et al. 2005) and is wide spread in the northern Atlantic (Kooistra et al. 2008), including polar coastal waters (Zhang et al. 2010). During the final stages of a *S. marinoi* bloom, PUA production was correlated with its abundance and cell lysis rates, suggesting a potential release of PUA into seawater whenever handling experimental containers. Surface seawater was collected by submerging a 20-L polycarbonate carboy.

Most diatom PUA research focused on inhibitory effects on reproduction and development of marine invertebrates such as copepods (Miralto et al. 1999; Ianora et al. 2004; Fontana et al. 2007; Lauritano et al. 2012; Brugnano et al. 2017). PUA can induce teratogenesis in some nauplii (Pohnert 2005) because these toxic metabolites selectively accumulate in the gonads of copepods (Wolfram et al. 2015). This phenomenon is considered an anti-grazer defense (Miralto et al. 1999; Ianora et al. 2004; Ianora et al. 2012). However, the extent of diatom-induced deleterious effects varies widely among copepod species and experiments (Leising et al. 2005b; Pierson et al. 2007; Sommer 2009; Jónasdóttir et al. 2011; Taylor et al. 2012). Flynn and Irigoien (2009) have proposed instead that the main target of PUA are the primary herbivores, microzooplankton. In addition, diatom-released PUA can affect phytoplankton community dynamics and structure by suppressing the growth of other species (Casotti et al. 2005; Ribai et al. 2007a).

These observations raise important questions about the role of diatom allelopathy in pelagic food webs. Specifically, little is known about the effects of PUA on microzooplankton and their trophic interactions with phytoplankton and planktonic copepods. Reduced or inhibited microzooplankton herbivory in the presence of senescent post-bloom diatoms (Sherr et al. 2009) and presumably cytotoxic *P. pouchetii* and *S. marinoi* was reported (Calbet et al. 2011; Stoecker et al. 2015). Dissolved PUA added at nearly ambient concentrations had an overall negative effect on natural microzooplankton growth rates, especially at the higher concentrations (Lavrentyev et al. 2015). However, the pronounced differences among common planktonic species indicate that PUA can induce changes in microzooplankton growth dynamic and community structure. Given the magnitude of carbon flux through the microbial food web (Buitenhus et al. 2010), determining PUA effects on microzooplankton trophic interactions is key to understanding the ecological role of these allelochemicals in the ocean.

The central question of our study “Does PUA production allows diatoms to escape grazing control by microzooplankton?” led to the following specific predictions: (H1): PUA inhibits phytoplankton growth; (H2): PUA impairs microzooplankton herbivory; (H3): PUA inhibits copepod herbivory; and (H4): PUA enhances copepod predation on microzooplankton. The goal of our study was to examine these hypotheses using natural marine plankton from productive coastal waters and dissolved PUA additions simulating a cytotoxic diatom bloom.

**Materials and methods**

Seawater for experiments was collected from the Choptank River, a tributary of the Chesapeake Bay, from the dock at the Horn Point Laboratory of the University of Maryland Center for Environmental Science (HPL, Cambridge, Maryland, Fig. 1) in April 2013. In May 2014, seawater was also collected near the Choptank River and additionally from the Chesapeake Bay at Ragged Point. In September 2014, seawater was collected from two Atlantic Ocean coastal locations: one near the Virginia Institute of Marine Science Eastern Shore Laboratory (ESL, Wachapreague, Virginia) and the other in Wachapreague Inlet. At each station, water temperature and salinity were measured using hand-held YSI probes (Model 30 and Pro2030DO).

Prior to sampling, all glassware, plastic containers, and tubing were soaked in 10% HCl and rinsed with copious amounts of deionized water and then seawater. Gloves were used whenever handling experimental containers. Surface seawater was collected by submerging a 20-L polycarbonate carboy.
During April 2013 and May 2014 experiments, the carboys were immediately transported to a temperature-controlled cold room at HPL, which was adjusted to ambient (± 1°C) water temperature. In September 2014, the water temperature was nearly equal to room temperature at ESL. All manipulations were conducted under dim light, and samples were stored in a closed cooler whenever not being handled. The collected water was carefully screened through a 200-µm mesh net to remove larger zooplankton such as copepods. Copepods were collected with a 50-cm diameter ring net fitted with 200-µm mesh that was towed vertically. The live sample was then diluted into 4-L containers filled with seawater from the site, and transported back to the shore-based lab in insulated coolers along with the seawater collected for the experiments. Copepods were sorted to species and life history stage under a stereomicroscope, examined for condition, and pipetted into small polycarbonate jars with filtered seawater before each experiment. Within 2 h of the initial collection, 20 copepods were transferred into 600-mL experimental bottles to achieve a concentration of 30 individuals L⁻¹. This concentration was chosen based on an approximate estimated clearance rate of 1 mL animal⁻¹ h⁻¹ by A. tonsa on diatoms (Berggreen et al. 1988; Saiz and Kiørboe 1995). All experiments were conducted using A. tonsa adults, and all animals used in the experiments were alive, swimming, and without any visible damage at the beginning of incubation.

Treatments included whole seawater (control), diluted seawater, whole seawater plus copepods, and the same treatments plus PUA additions. Triplicates of control and PUA treatments were prepared by carefully adding the experimental water to 0.61-L Nalgene clear polycarbonate bottles. Phytoplankton growth and grazing loss rates were determined in two-point dilution assays (Landry et al. 2008). In contrast to the original dilution method, this approach does not rely on a linear model. The grazing rate (g) is calculated as the difference in prey growth (µ) in undiluted and highly diluted treatments. The latter treatment approximates “zero” grazing. We selected this approach because it provides the same growth and grazing rate estimates as the original multi-point approach (Strom and Fredrickson 2008; Chen 2015) even when nonlinear responses are taken into account (Morison and Menden-Deuer 2017). It is also more efficient and less affected by uncertainty in changes in microzooplankton clearance and growth rates, and trophic interactions across a dilution gradient (Dolan et al. 2000; First et al. 2007; Calbet and Saiz 2013). The triplicated diluted treatments were prepared by mixing filtered seawater (0.2 µm large volume Pall Science pleated capsules using gravity flow) with whole seawater to reach a final 5% whole seawater treatment. The capsules were thoroughly rinsed with deionized water prior to use.

To equalize plankton growth conditions across different treatments, all samples were amended with L1 (Guillard and Hargraves 1993) growth media to the final concentrations of 8.5 µM, 0.3 µM, and 1.0 µM (N, P, Si, respectively). The PUA treatments were prepared by adding a methanol solution of trans-2,4-octadienal (OD, W372102, Sigma-Aldrich, Saint Louis, Missouri, U.S.A.) and trans-2,4-heptadienal (HD, 180548, Sigma-Aldrich, Saint Louis, Missouri, U.S.A.) at the OD : HD ratio of 1 : 10. In Exp. 1, 4, and 5 experimental samples were inoculated with 5 nM of HD and 0.5 nM of OD (final concentration). While in Exp. 2 and 3, the concentrations of HD and OD were 2.0 nM and 0.2 nM, respectively. The concentrations of PUA and the OD : HD ratio used were meant to reproduce the conditions experienced by plankton in productive coastal waters during a bloom of PUA-producing diatoms (Vidoudez et al. 2011; Ribalet et al. 2014). The final concentration of methanol in seawater was 0.10 µL L⁻¹ and 0.21 µL L⁻¹, respectively. The effect of methanol was tested and no negative effect on plankton growth was observed (Lavrentyev et al. 2015). The bottles were closed with caps lined with plastic wrap to prevent air headspace. In Exp. 1–3, the bottles were mounted on a plankton wheel (~ 0.25 rpm) and incubated for 24 h under ambient seawater temperature in a walk-in incubator.

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The light: dark cycle maintained with cool white fluorescent growth lights was 14:10 h. Daily light integral (DLI) measured using a LI-COR spherical quantum sensor was 1.0 mol m$^{-2}$d$^{-1}$. The light level was chosen to mimic that in the turbid Choptank River. In Exp. 4–5, bottles were incubated at the ESL dock at 0.5 m depth and covered with two layer of black window screen. Due to the cloudy weather and screening, DLI was approximately 2.0 mol m$^{-2}$ d$^{-1}$.

Plankton samples were collected at initial and final (24 h) time points. In the beginning, triplicated samples from the 200-µm screened seawater were collected before it was added to experimental bottles. For chlorophyll a (Chl) analysis, 50–150 mL of seawater was filtered onto 0.7 µm 25 mm Whatman GF/F filters. Two subsamples were collected and processed from each triplicated sample for quality assurance and then averaged. Chl was extracted in 90% acetone for 24 h at −20°C and measured using the acidic method (Arar and Collins 1997) on a Turner Designs 10AU fluorometer (except Exp. 1, where the nonacidic method (Welschmeyer 1994) and a Turner Designs TD-700 fluorometer were used). Phyt plankton for flow-cytometry were preserved with 0.2 µm-filtered formaldehyde (1% final concentration), shock-frozen in liquid N$_2$, and subsequently processed using a Partec CyFlow SL flow cytometer equipped with a 50 mW 488 nm blue solid-state laser. Two 1 mL subsamples were collected and processed from each triplicated sample for quality assurance and then averaged. For processing larger volumes from diluted samples, we used an external PC-controlled New Era N-1000 syringe pump. Optical properties of cells were compared using forward angle light scatter (FSC) and right-angle light scatter (SSC). Red (FL3) and orange (FL2) fluorescence was used as a proxy for Chl and phycocyrin cell content. Fluorescent beads (1 µm) were added as internal standard to each sample. The obtained data were analyzed using the FlowJo v.10.2 software package. In addition, phytoplankton were analyzed via microscopy as described below.

Triplicated 100-mL microplankton samples were collected from the carboy with seawater in the beginning of incubation and one from each experimental bottle in the end. The samples were preserved in 2% (final concentration) acid Lugol’s iodine, stored at 4°C in opaque containers, and post-fixed with 1% (final concentration) formaldehyde after 24 h. Plankton were settled in 10–25 mL Utermöhl chambers and counted under an Olympus IX-70 inverted microscope equipped with differential interference contrast (DIC), epifluorescence, and a digital camera. Although dominant plankton were abundant enough to be counted in random fields at 400X, some larger microzooplankton were less numerous and required larger volumes. To count them, the entire surface area of a chamber was scanned at 200X. In some cases, we settled 1–3 additional aliquots to count these cells; the counts from sub-samples were then combined. The subsample volume and number varied among experiments but were always the same in a given experiment. Protists were identified and classified as described in Lavrentyev et al. (2015). Microzooplankton biovolumes were calculated from their linear dimensions by approximating geometric shapes (Sun and Liu 2003) and converted to carbon (Menden-Deuer and Lessard 2000). Tintinnid volumes were calculated based on their cell dimensions; empty loricas were disregarded.

Prey apparent growth rates were calculated assuming exponential growth: $\mu = \ln(N_t/N_0)/(t/24)$, where: $\mu$: growth rate (d), N0 and Nt = prey abundance at the beginning and end of the experiment, respectively, and t = time (hours). Grazing mortality rates (g) were determined as the difference between $\mu$ measured in the diluted (µ5%) and whole (µWSW) seawater samples: $g = \mu_{5\%} - \mu_{WSW}$. In the experiments where prey grew slower in diluted samples, or not significantly different from the whole seawater treatment, g was not calculated and reported as 0 (Stoecker et al. 2014), whereas the growth of prey was reported from the whole seawater treatment. Data are presented as mean ± SD throughout text.

The obtained data were analyzed via Student two-tailed t-test for independent samples assuming unequal variance and using Minitab 17. Standard deviation was used as a measure of dispersion throughout the study, including the figures. First, we compared initial and final plankton concentrations (both Chl a and cell counts). Second, test was performed to compare prey growth rates between diluted and whole seawater treatments. Only when plankton abundance (and/or Chl) changed significantly (at the 95% confidence level) over time, and faster in diluted than in whole seawater treatments, we calculated microzooplankton grazing rates, which were subsequently compared between control and PUA treatments. Copepod clearance (CR) on Chl, ciliates, and dinoflagellates were calculated using Frost’s (1972) equations.

Results

During the spring (April–May) experiments in the Chesapeake Bay (Exp. 1–3), the surface water temperature and salinity (15.7 ± 1.5°C, 9.8 ± 0.3 PSU, Table 1) were lower compared to the late September experiments (Exp. 4–5) in the Atlantic coastal waters near ESL (23.6 ± 0.5°C, 29.9 ± 0.1 PSU). Chl concentrations ranged from 10.4 ± 1.00 µg L$^{-1}$ to 1.13 ± 0.04 µg L$^{-1}$ with both extreme values recorded in the Chesapeake Bay (Exp. 1 and 3, respectively). At the Atlantic sites, Chl averaged at 4.98 ± 1.14 µg L$^{-1}$.

The plankton composition at the experimental sites has been described in detail elsewhere (Lavrentyev et al. 2015). Briefly, differences in Chl concentration corresponded to different plankton assemblages (Fig. 2). The plastidic species *Procentrum minimum* and *Karlodinium veneficum* (formerly known as *K. micrum*) were dominant microzooplankton in terms of numbers and biomass in all Chesapeake Bay experiments (Exp. 1–3). The peridiniid dinoflagellate *Diplopsalis lenticula* also was an important microzooplankton biomass contributor in Exp. 1.
Ciliate assemblage was formed by small and medium-sized heterotrophic species including *Lohmanniella oviformis*, *Strombidium* sp., *S. epidemum*, *Balanion comatum*, and *Tintinnopsis nana*. The latter two heterotrophic species contributed disproportionately to ciliate biomass. The diatoms *Pseudo-nitzschia delicatissima* and *Mesodinium rubrum* dominated numerically among pigmented cell > 5 μm in Exp. 1 (ca. 70%) and *Thalassiosira* sp. (cf. *pseudo-nana*) in Exp. 2 (ca. 40%). The plastidic *Mesodinium rubrum* and heterotrophic *Pelagostrobilidium neptuni* and *Uroticina* sp. formed ciliate biomass in Exp. 2. At the Ragged Point (Exp. 3), cryptophyte flagellates were numerically dominant and diatoms (*Navicula rhynchocephala* and *Cymbella* sp.) contributed ~ 25% to the >5 μm fraction. At this site, the large (ca. 100 μm) heterotrophic *Prorodon* sp. formed ciliate biomass along with *T. nana* and *L. ovifromis*.

In the Atlantic coast waters, diatoms were more abundant (~ 80% of the >5 μm fraction) and diverse. Their assemblage was dominated by *Chaetoceros* spp., *R. stolforthii*, *Pseudo-nitzschia* sp., and *Ditylum* sp. (cf. *brightwellii*). Dinoflagellate biomass was dominated by the heterotrophic *Gyrodiunium spirale* and *Gymnodinium* sp. and the plastidic *Heterocapsa rotundata*. The heterotrophs *Favella panamensis* and *P. neptuni* dominated ciliate biomass along with the mixotrophic *Cyclostichum gigas*. The copepod *A. tonsa* dominated the net collected mesozooplankton at all sites.

The Chl-based phytoplankton growth rates (μ) were measured in all experiments and displayed differences among locations (Fig 3a). In the Chesapeake Bay experiments phytoplankton grew slower (0.44 ± 0.06 d⁻¹ and 0.59 ± 0.00 d⁻¹) than at the Atlantic sites (1.52 ± 0.07 d⁻¹ and 1.48 ± 0.10 d⁻¹, in Exp. 4 and 5, respectively). PUA additions had no effect on phytoplankton growth. Microzooplankton herbivory (Fig. 3b) was measurable in all experiments and followed the same trend as the growth rates: lower in the Chesapeake Bay (0.35 ± 0.14 d⁻¹) and higher in the Atlantic sites (0.86 ± 0.22 d⁻¹). When exposed to PUA, microzooplankton herbivory significantly declined compared to control in Exp. 2, 4, and 5. The maximum reduction of 60% was observed in Exp. 2 and the grazing rate decreased from 0.22 ± 0.05 to 0.09 ± 0.05 d⁻¹. In Exp. 4 and 5, reduction was of about 30% (1.02 ± 0.14 vs. 0.74 ± 0.04 vs. 0.48 ± 0.07 d⁻¹). No statistical difference was observed for the grazing rates in the PUA and control treatments in Exp. 1 and 3.

Three distinct cytometric populations were discriminated based on their scatter and fluorescence patterns (Fig. 4). The first group (Syn) included *Synechococcus*-like picoplankton cells, the second (Euk < 5 μm)—small eukaryotic cells, and
the third (Euk > 5 μm) included diatoms and other larger phytoplankton. All three groups displayed higher growth rates at the Atlantic sites compared to the Chesapeake Bay (Fig. 5 a,c,e). Syn grew on average at 0.15 ± 0.09 d⁻¹ in the first three experiments and 1.20 ± 0.28 d⁻¹ in the last two. Likewise, Euk < 5 μm and Euk > 5 μm presented much lower growth rates at Chesapeake Bay sites (0.18–0.61 d⁻¹) compared to the Atlantic sites (1.17–1.68 d⁻¹). None of the three groups responded to PUA in terms of growth (t-test; p > 0.05).

In control, microzooplankton grazing on Syn varied between 0.25 ± 0.04 d⁻¹ and 1.49 ± 0.07 d⁻¹, except Exp. 3 where no grazing was detected (Fig.5b). No grazing on Euk < 5 μm (Fig. 5d) occurred in the Chesapeake Bay experiments, whereas at the Atlantic coast sites, these cells were grazed at 0.30 ± 0.12 d⁻¹. The grazing rates on Euk > 5 μm ranged from 0.07 ± 0.03 d⁻¹ in Exp. 3 to 0.73 ± 0.00 d⁻¹ in Exp 1 (Fig. 5f).

PUA affected grazing on the three groups differently. For Syn grazing significantly increased in three experiments out of five. In contrast, PUA reduced or even inhibited grazing on Euk > 5 μm. Finally, no difference between the PUA treatment and control was detected for grazing on Euk < 5 μm.

In control treatments, A. tonsa preferentially ingested ciliates by clearing them at rates of 11.7 ± 5.04 mL animal⁻¹ d⁻¹ in Exp. 4 to 35.9 ± 8.94 mL animal⁻¹ d⁻¹ in Exp.3 (Fig. 6). These rates were twice those measured on dinoflagellates (9.01 ± 4.02 to 12.7 ± 4.93 mL animal⁻¹ d⁻¹, in Exp. 1 and 5, respectively) and about an order of magnitude higher than on Chl (1.31 ± 0.00–6.29 ± 2.46 mL animal⁻¹ d⁻¹). In Exp. 2 and 4, A. tonsa ingested only the ciliates. However, when exposed to PUA it also started feeding on phytoplankton and dinoflagellates. The addition of hepta- and octadie-nal significantly stimulated copepod feeding rates in all experiments but one (Exp. 1). Again, the strongest response to PUA was observed for CR on ciliates (Fig. 6). Despite their low biomass in Exp. 2, mixotrophic ciliates were cleared (mL animal⁻¹ d⁻¹) at 79.8 ± 10.1 in the presence of PUA vs. 0 ± 0.8 in control (p = 0.04). In Exp. 3 this effect was also significant: 31.2 ± 0.7 PUA vs. 0 ± 1.7 control (p < 0.01). Their heterotrophic counterparts were cleared (mL animal⁻¹ d⁻¹) at 23.7 ± 5.1 PUA vs. 0 ± 0.8 control (p = 0.05) in Exp. 1, and 65.0 ± 3.5 PUA vs. 41.2 ± 5.4 control (p =0.05) in Exp. 5. At the species-specific level, the copepods consumed a variety of cells ranging from 10 μm to 100 μm, both ciliates and dinoflagellates (Fig. 7). In many cases, the species-specific clearance rates were also enhanced after PUA additions. Furthermore, we could not calculate clearance rates on several microzooplankton species, because they became too scarce to be counted in the copepod + PUA treatment (Fig. 8).

Discussion

PUA concentrations

The goal of this study was to simulate PUA effects on plankton during a bloom of cytotoxic diatoms in productive coastal waters. The dissolved PUA final concentrations added to experimental bottles corresponded to 2.7 and 6.7 × 10⁶ diatom cells L⁻¹ in Exp. 2, 3 and 1, 4, 5, respectively, based on PUA production of 7.5 fmol cell⁻¹ by the cytotoxic strain of S. marinoi (Ribalet et al. 2007b) and the assumption that 10% of cells undergo lysis and release PUA (Balestra et al. 2011). In the natural waters, PUA-producing diatoms can reach the abundance of 10⁷ cells L⁻¹ (Casotti et al. 2005; Ribalet et al. 2014). Dissolved PUA concentrations measured in the northern Adriatic Sea during the S. marinoi blooms ranged from 2.53 nM to 28.3 nM (Vidoudez et al. 2011; Ribalet et al. 2014). Thus, the PUA concentrations used in this study are ecologically realistic.

The background PUA concentrations remained low during this study in both the Chesapeake Bay and Virginia coastal locations (combined dissolved and particulate Octa- and Heptadienal, 0.01–0.08 nM, Lavrentyev et al. 2015), indicating that no cytotoxic bloom related to PUA was in progress at the time of experiments. However, these data also suggest that such conditions could have occurred at an earlier date. Specifically, the total concentration of PUA at the Atlantic coastal sites (Exp. 4 and 5) were close to the residual level of 0.1 nM that can persist in the water column following a cytotoxic bloom event (Vidoudez et al. 2011). The experimentally determined PUA disappearance times are temperature-dependent and vary between 200 h and 60 h at 10°C and 20°C, respectively (Bartual and Ortega 2013). Although there is no other published information on PUA in the Chesapeake Bay and the Virginia coast...
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Allelopathy-induced trophic cascades

Fig. 5. Growth (a, c, e) and grazing loss (b, d, f) rates of phytoplankton populations from Fig. 4 in dilution experiments. Bars = ± 1 standard deviation; numbers = p-values of Student t-test; n.d.—not detected (i.e., not significantly different from zero based on t-test).
coastal waters, *Skeletonema* is common in both ecosystems and occasionally forms blooms. For comparison, in the Strait of Gibraltar (Morillo-Garcia et al. 2014) and the offshore Atlantic Ocean waters (Bartual et al. 2014), PUA concentrations were in the pico-molar range under no-bloom conditions.

**Microplankton response**

Higher temperature and better light conditions could have contributed to the observed difference in the phytoplankton growth rates between the Chesapeake Bay (Exp. 1–3) and Virginia coastal waters (Exp. 4–5). Nutrients were added to all treatments at the levels that were above the half-saturation constants of phytoplankton N and P uptake in the Chesapeake Bay (Fisher et al. 1992). Therefore, they would have relieved nutrient limitation, had it existed prior to the incubation. Given the ambient nitrate and phosphate concentrations in the Chesapeake Bay (19 μM dissolved inorganic nitrogen and 0.11 μM orthophosphate; Chesapeake Bay Program, http://www.chesapeakebay.net), these additions were unlikely to be responsible for the phytoplankton growth. We do not have similar data for the Virginia waters and, therefore, cannot exclude the possibility of nutrient-enhanced growth. Importantly, the growth conditions were the same for control and PUA treatments in any given experiment.

Despite the pronounced differences in plankton dynamics and composition, the response of phytoplankton to PUA additions was similar among the experiments; we did not observe any adverse direct effects of dissolved PUA on phytoplankton growth in terms of either Chl concentration or cell numbers. Our H1 is hence rejected. Similarly, in a natural system, no significant changes in Chl were observed in PUA vs. control treatments (Paul et al. 2012). Depending on the amount of PUA produced and released in the water, cells can develop resistance to successive exposure or undergo cell lysis (Vidoudez and Pohnert 2008). In contrast, the growth of several cultured marine diatoms and a cytotoxic dinoflagellate was suppressed by exposure to much higher (6 μM to 36 μM, i.e., 1000–18,000X) PUA concentrations (Hansen and Ellertsen 2006; Casotti et al. 2005; Ribalet et al. 2007a; Pichierri et al. 2016). The effects of nearly ambient concentrations of allelochemicals on phytoplankton may take longer than 24 h to develop. For example, prolonged exposure to low (nM) concentrations of PUA have caused similar effects in cultured diatoms as short-term doses of much higher (μM) concentrations (Dittami et al. 2010).

It is noteworthy therefore that the relatively low PUA concentrations triggered such a strong response at the consumer level in our study. Dissolved PUA additions reduced the growth rates of microzooplankton herbivory based on changes in total Chl, thus supporting H2. Herbivory, as measured in dilution experiments, is a community process and Chl may not be an ideal proxy for complex trophic interactions involving multiple taxa. The strongest decrease in the FCM-based grazing rates was observed on Euk > 5 μm, which included diatoms. At the same time, the grazing pressure on smaller phytoplankton such as *Synechococcus* increased in four out of five experiments.

Since the diatoms in our study did not produce appreciable amounts of PUA at the time of experiments, the observed effects on herbivory were solely due to the added dissolved PUA. These results suggest that microzooplankton, possibly based on their chemosensory abilities, can adjust their
feeding behavior to reduce exposure to potentially toxic prey. A similar behavior was observed in three pelagic ciliate species exposed to a mixed diet of the toxic raphidophyte Heterosigma akashiwo and nontoxic prey (Graham and Strom 2010). Thus, our results support the idea that oxylipin production in diatoms is a defense mechanisms against herbivorous microzooplankton (Flynn and Irigoien 2009). Consumption of dominant phytoplankton by grazers may

Fig. 7. Copepod clearance rates on abundant species of ciliates (C) and dinoflagellates (D) in each experiment. PRM = Prorocentrum minimum (D, 18 μm), BPL = Balanion planktonicum (C, 10 μm), LOV = Lohmaniella oviformis (C, 15 μm), MRB = Mesodinium rubrum (C, 30 μm), KVE = Karlodinium veneficum (D, 13 μm), URO = Urotricha sp. (C, 18 μm), HGR = Halteria grandiniella (C, 40 μm), PSN = Pelagostrobilidium neptuni (C, 50 μm), TLV = Tintinnopsis levigata (C, 30 μm), TNN = T. nana (C, 12 μm), GYM1 = Gymnodinium sp. 1 (D, 25 μm), GYM2 = Gymnodinium sp. 2 (D, 40 μm), STE = Strombidium epidermum (C, 18 μm). Bars = ± 1 standard deviation; numbers = p-values of Student t-test; n.d.—not detected (i.e., not significantly different from zero).
create an opening for secondary species (Kiørboe et al. 1996). Likewise, it appears that PUA-producing species can reduce the population size of their competitors indirectly via re-directing microzooplankton toward them. Most lipid precursor molecules are converted to PUA within minutes via re-directing microzooplankton toward them. Most lipid precursor molecules are converted to PUA within minutes. Thus, we can accept H4. At the species-specific level, PUA-stimulated clearance was even more apparent.

The exact mechanisms of PUA-enhanced copepod predation on microzooplankton remain to be determined. PUA can inhibit the flagellar activity of starfish sperm cells (Caldwell et al. 2004) and the growth and swimming speed of the harmful dinoflagellate *Cochlodinium polykrikoides* was inhibited by filtrates from the cultures of marine diatoms *Skeletonema costatum*, *Thalassiosira weissflogii*, *Chaetoceros danicus* and a surprising increase in egg production rate was not reported deleterious effect of diatoms on copepod reproduction may be due to experimental conditions when copepods are confronted with a toxic monoculture as the only source of food (Sommer 2009). In fact, several field studies have shown that copepods can reject the most abundant phytoplankton, particularly certain PUA-producing species (Leising et al. 2005b).

The feeding response of *A. tonsa* on slow-growing cells of *Thalassiosira weissflogii* were enhanced by the addition of cell-free aliquots of algal exudate from the fast growing cells (Cowles et al. 1988). This copepod was able to locate patches of *S. marinoi* and *T. weissflogii* (Amin et al. 2011; Tiselius et al. 1993), possibly relying on chemical cues released by these diatoms. In the presence of PUA, ciliates were consumed two-three times the rates in control and up to ten times faster than dinoflagellates. Thus, we can accept H4. At the species-specific level, PUA-stimulated clearance was even more apparent.

The effects of PUA on microzooplankton growth dynamics may reinforce the above trophic response. In the concomitant study, abundant ciliates and dinoflagellates demonstrated a gradient of tolerance to PUA from a distinctly inhibitory reaction even at the lowest concentration to no response at all (Lavrentyev et al. 2015). Overall, the direct cytotoxic effect of PUA on microzooplankton was mild; a number of species responded to PUA only at 20 nM, which corresponds to a heavy bloom. It is noteworthy that the dinoflagellate *G. spirale*, a common voracious diatom feeder, was one of the most affected by PUA species. In addition, the strong antimotic effect of PUA on the large raptorial ciliate *Prorodon* sp. likely led to an increase in the growth of small-sized ciliates and dinoflagellates such as *L. oviformis* and *P. minimum*, which typically feed on pico- and nanoplankton (Jönsson 1986; Burkholder et al. 2008). Thus, oxylipin production by diatoms appears to have triggered cascading effects within the microzooplankton community resulting in a more diatom-friendly structure.

**Mesozooplankton response**

In contrast to microzooplankton, *A. tonsa* increased clearance rates on phytoplankton in the PUA treatments (with the exception of Exp. 1), thus forcing us to reject H3. The odor of phytoplankton may repulse (Jüttnerv 2005) or attract pelagic crustaceans (Maibam et al. 2015). The copepod *Temora stylifera* increased *P. minimum* ingestion rates in culture when exposed to 0.5 μM to 2 μM of 2E, 4E-decadienal (Ka et al. 2014). The reason for the enhanced feeding rates and a surprising increase in egg production rate was not clear. The authors also reported that *T. stylifera* was attracted to decadienal when it was incorporated into an agarose gel, suggesting that it recognized PUA as a food-related signal. Chemosensory responses are common among copepods (DeMott and Watson 1990). For example, the ingestion rates of *A. tonsa* on slow-growing cells of *Thalassiosira weissflogii* were enhanced by the addition of cell-free aliquots of algal exudate from the fast growing cells (Cowles et al. 1988). This copepod was able to locate patches of *S. marinoi* and *T. weissflogii* (Amin et al. 2011; Tiselius et al. 1993), possibly relying on chemical cues released by these diatoms. In the presence of PUA, ciliates were consumed two-three times the rates in control and up to ten times faster than dinoflagellates. Thus, we can accept H4. At the species-specific level, PUA-stimulated clearance was even more apparent.

The exact mechanisms of PUA-enhanced copepod predation on microzooplankton remain to be determined. PUA can inhibit the flagellar activity of starfish sperm cells (Caldwell et al. 2004) and the growth and swimming speed of the harmful dinoflagellate *Cochlodinium polykrikoides* was inhibited by filtrates from the cultures of marine diatoms *Skeletonema costatum*, *Thalassiosira weissflogii*, and *Chaetoceros danicus* (Lim et al. 2014). Although no data exists on PUA effects on ciliate swimming activities, a similar effect potentially could expose the affected microzooplankton to increased predation risk. However, the observed increase in picoplankton grazing mortality after PUA addition may indicate that ciliate motility was not impaired, at least at the nM concentration used in our study. In addition, the disappearance of such PUA-resistant ciliates as *C. gigas*, *M. rubrum*, and *Strombidium conicum* (Lavrentyev et al. 2015) in the copepod + PUA treatment may indicate that PUA directly influenced *Acartia* feeding.

Whether it is driven by chemical cues or reduction in the escape ability of their prey, the feeding response of *A. tonsa* in this study is consistent with the idea that copepod predation on microzooplankton can reduce grazing pressure on diatoms. However, it does not support the contention that PUA acts as a grazer deterrent against copepods. The frequently reported deleterious effect of diatoms on copepod reproduction may be due to experimental conditions when copepods are confronted with a toxic monoculture as the only source of food (Sommer 2009). In fact, several field studies have shown that copepods can reject the most abundant phytoplankton, particularly certain PUA-producing species (Leising et al. 2005b). On the other hand, *Calanus finmarchicus* fed on *S. marinoi* during its mesocosm bloom (Ray et al. 2016). Predation had a stronger effect on the recruitment...
success of *Calanus* than diatom allelopathy throughout the spring bloom conditions (Pierson et al. 2007).

In general, copepod population growth is much more sensitive to variations in mortality than to variations in fecundity (Kiørboe 1997). Using a modeling approach, Flynn and Irigoien (2009) have demonstrated that killing the copepod offspring cannot be sustained as a defense mechanism since the probability of the copepod offspring consuming a cell of the same clone that induced hatching inhibition is lower than that of consuming a competitor or predator of that clone. Thus, PUA production would not confer any advantage to the diatoms if their main targets were copepod eggs. While the anti-proliferation effect of PUA on copepod reproduction has been demonstrated in multiple studies, it may be simply “collateral damage” in the chemical warfare against the main herbivores, microzooplankton.

In our subsequent experiments, additions of a cultured cytotoxic *S. marinoi* strain to seawater led to an increase in predation on *M. rubrum* and *Gymnodinium* sp. by *Centropages hamatus* (unpublished). It remains to be seen whether direct encounters with natural populations of cytotoxic diatoms result in the same effects on the diatom-microzooplankton-copepod link as dissolved PUA. In this study, hepta- and octadienal were dissolved in a relatively large volume compared to the average microplankton cell size. Local concentrations in the immediate vicinity of PUA-producing diatom cells could be much higher due to a low diffusion process away from a non-swimming producer (Ribalet et al 2007a). It should be noted, however, that in the water column, PUA dissipation and decay rates might be quite different from those in a culture. In this study, we tested the effects of dissolved PUA on random plankton assemblages that may or may not have experienced a real-life bloom of PUA-producing diatoms prior to our experiments. Further research is needed to test whether plankton communities occurring during such blooms will respond to PUA similarly.

Natural plankton communities may include multiple diatom and non-diatom populations producing a variety of bioactive secondary metabolites (e.g., Strom 2008; Ianora et al. 2012; Schwartz et al. 2016). For example, *S. marinoi* growth was inhibited in the presence of a domoic acid-producing strain of *P. delicatissima* (Prince et al. 2013). Interestingly, microzooplankton herbivory and growth were not affected by this cytotoxin (Olson and Lessard, 2010). On the other hand, we observed a complete inhibition of microzooplankton herbivory in the Chesapeake Bay during a bloom of the diatom *Leptocylindrus danicus* even though no PUA production was detected at the time of the bloom (unpubl.). These observations demonstrate the complexity of trophic and chemical interactions, which present an obvious challenge in field PUA experiments with natural plankton. Thus, our conceptual model (Fig. 9), which illustrates how diatom-
produced secondary metabolites may alter the lower food web, is necessarily simplified. As more data become available, this model can be expanded and modified.

Conclusions

To our knowledge, this study is the first attempt to experimentally examine the effects of diatom-linked PUA on the critical phytoplankton-microzooplankton-copepod link. Its results demonstrate that PUA may trigger complex cascading effects in the pelagic food web. In particular, PUA appears to act as deterrent for microzooplankton herbivory on diatoms, while enhancing herbivory on small, more palatable phytoplankton. This switch in microzooplankton feeding behavior and the corresponding shifts in grazer composition should favor PUA-producing diatoms by simultaneously reducing direct grazing losses and competition. Additionally, increased copepod predation on microzooplankton, particularly on ciliates, should further advantage PUA-producing diatoms by reducing primary herbivore numbers. Combined, these effects could create a “grazing loophole” (Irigoien et al. 2005) that opportunistic diatoms would be able to exploit under at least partially favorable abiotic conditions. At the ecosystem level, PUA allelopathy should lead to the increased vertical transport of diatom carbon, which otherwise would be recycled through the microbial food web.

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Acknowledgments

The authors are grateful to Adrianna Ianora, Raffaella Casotti, and Suzanne Strom for helpful discussion at the planning stages of this study, Nicole Millette and Chase Altman for laboratory assistance, the ESL staff for their assistance during the September 2014 experiments, and the anonymous reviewers for helpful criticisms. Plankton symbols used in Fig. 9 are courtesy of the Integration and Application Network, University of Maryland Center for Environmental Science. This study was funded by the National Science Foundation (Award OCE-1357168/1357169).

Conflict of Interest

None declared.

Submitted 19 April 2017
Revised 29 July 2017
Accepted 17 October 2017

Associate editor: Ronnie Glud